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**Tomato Glutamate Decarboxylase Genes *SlGAD2* and *SlGAD3* Play Key Roles in Regulating γ-Aminobutyric Acid Levels in Tomato (*Solanum lycopersicum*)**

**GABA accumulation in tomato fruits via *GAD***

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**Abbreviations**

**AZ, azygous; CaM, calmodulin; CaMV, cauliflower mosaic virus; DW, dry weight; FW, fresh weight; GABA, γ-aminobutyric acid; GABA-T, GABA transaminase; GAD, glutamate decarboxylase; HO, homozygous; MG, mature green; MST, mass spectral tag; qRT-PCR, quantitative reverse transcription-PCR; SSA, succinic semialdehyde; SSADH, succinic semialdehyde dehydrogenase; TCA, tricarboxylic acid; WT, wild-type**

**Abstract**

Tomato (*Solanum lycopersicum*) can accumulate relatively high levels of γ-aminobutyric acid (GABA) during fruit development. However, the molecular mechanism underlying GABA accumulation and its physiological function in tomato fruits remain elusive. We previously identified three tomato genes (*SlGAD1*, *SlGAD2* and *SlGAD3*) encoding glutamate decarboxylase (GAD), likely the key enzyme for GABA biosynthesis in tomato fruits. In this study, we generated transgenic tomato plants in which each *SlGAD* was suppressed and those in which all three *SlGADs* were simultaneously suppressed. A significant decrease in GABA levels, i.e., 50–81% compared with wild-type (WT) levels, was observed in mature green (MG) fruits of the *SlGAD2*-suppressed lines, while a more drastic reduction (up to <10% of WT levels) was observed in the *SlGAD3*- and triple *SlGAD*-suppressed lines. These findings suggest that both *SlGAD2* and *SlGAD3* expression are crucial for GABA biosynthesis in tomato fruits. The importance of *SlGAD3* expression was also confirmed by generating transgenic tomato plants that over-expressed *SlGAD3*. The MG and red fruits of the over-expressing transgenic lines contained higher levels of GABA (2.7- to 5.2-fold) than those of the WT. We also determined that strong down-regulation of the *SlGADs* had little effect on overall plant growth, fruit development or primary fruit metabolism under normal growth conditions.

**Keywords**

Fruit, GABA, GAD, Molecular breeding, RNAi, Tomato

## Introduction

γ-Aminobutyric acid (GABA) is a ubiquitous non-protein amino acid that functions as a main inhibitory neurotransmitter in the mammalian central nervous system (Owens and Kriegstein 2002). Oral administration of GABA provides various benefits to human health, such as lowering blood pressure (Inoue et al. 2003; Kajimoto et al. 2004) and producing relaxation effects (Abdou et al. 2006). Therefore, GABA has received much attention as a health-promoting functional compound, and several GABA-enriched foods have been commercialized to date (Tsushida et al. 1987; Kajimoto et al. 2004). GABA has been detected in many plant tissues, such as shoots, roots, nodules, cultured plant cells, tubers, flowers and fruits (Sulieman 2011). Although the physiological function(s) of GABA in plants has not yet been fully defined, many suggest that GABA and its metabolic pathway are involved in pollen tube growth (Palanivelu et al. 2003; Yu et al. 2014), defense against pests and pathogen attacks (Bown et al. 2006; Seifi et al. 2013), regulation of ROS production (Shi et al. 2010; Liu et al. 2011) and cell elongation (Renault et al. 2011). Rapid and drastic increases in GABA levels have also been observed in various plant tissues in response to many diverse stimuli, including heat shock, mechanical stimulation, hypoxia and phytohormones (Bown and Shelp 1997; Shelp et al. 1999).

In higher plants, GABA is mainly metabolized via a short pathway known as the GABA shunt (Satya-Narayan and Nair 1990; Bouché and Fromm 2004a). The GABA shunt bypasses two steps (the oxidation of α-ketoglutarate to succinate) of the tricarboxylic acid (TCA) cycle via reactions catalyzed by three enzymes; glutamate decarboxylase (GAD), GABA transaminase (GABA-T) and succinic semialdehyde dehydrogenase (SSADH). The first enzyme, GAD, catalyzes the irreversible decarboxylation of glutamate to produce GABA and $CO_2$. GABA is then reversibly transaminated by

the second enzyme, GABA-T, to form succinic semialdehyde (SSA), which is subsequently oxidized by the third enzyme, SSADH, to produce succinate. The resulting succinate then flows into the TCA cycle. The GABA shunt is considered to play a major role in carbon and nitrogen primary metabolism and to be an integral part of the TCA cycle under stress and non-stress conditions (Fait et al., 2008).

GAD is widely found in the plant kingdom (Satya-Narayan and Nair 1990) and has been identified in various higher plants. Plant GAD is a typical pyridoxal-5′-phosphate (PLP)-dependent enzyme that exists in hexameric forms (Gut et al. 2009). Unlike its counterparts in animals and bacteria, plant GAD possesses an additional C-terminal 30 to 50 residues, known as the calmodulin (CaM)-binding domain (CaMBD) (Baum et al. 1993; Yap et al. 2003). According to *in vitro* studies, GAD activity is stimulated by low pH (<6.0) and the binding of $Ca^{2+}$/CaM to CaMBD under physiological pH (Snedden et al. 1996). In addition, transgenic studies show that the removal of CaMBD results in higher GABA accumulation in plants (Baum et al. 1996). Therefore, CaMBD is thought to activate the GAD enzyme in the presence of $Ca^{2+}$/CaM, such as under stress conditions, leading to an increase in cytosolic $Ca^{2+}$ concentrations, and it also functions as an autoinhibitory domain in the absence of $Ca^{2+}$/CaM, such as under non-stress conditions. However, in recent decades, new types of plant GADs have been identified in rice and apple fruits (Akama et al. 2001; Akama and Takaiwa et al. 2007; Trobacher et al. 2013). Both rice GAD2 (OsGAD2) and apple GAD3 (MdGAD3) possess the C-terminal extensions without the CaMBD, thereby exhibiting $Ca^{2+}$/CaM-independent enzyme activities. Although the C-terminal extension of OsGAD2 still functions as an autoinhibitory domain, that of MdGAD3 does not play such a role, and this enzyme is constitutively active. Thus, plant GADs appear to have multiple regulatory modes of activation depending on their functions.

Tomato is a major crop that accumulates relatively high levels of GABA in its fruits (Matsumoto et al. 1997). The GABA levels in tomato fruits change drastically during fruit development, with GABA levels increasing from flowering to the mature green (MG) stage and then rapidly decreasing during the ripening stage (Rolin et al. 2000; Akihiro et al. 2008). Although GABA comprises up to 50% of the free amino acids at the MG stage, the molecular mechanism underlying GABA accumulation and the physiological functions of GABA during tomato fruit development remain unclear. The tomato genome contains at least three *GAD* genes (*SlGAD1*, *SlGAD2* and *SlGAD3*) that function during fruit development. The mRNA levels of *SlGAD1* (also known as *ERT D1*) are high when the fruit matures, while the mRNA levels of *SlGAD2* (most likely allelic to *GAD-19* based on sequence identity) and *SlGAD3* are highest during early fruit development and decline as the fruit matures (Akihiro et al. 2008; Gallego et al. 1995; Kisaka et al. 2006). Previously, Kisaka et al. (2006) reported that transgenic tomato plants in which *GAD-19* (allelic to *SlGAD2*) is suppressed exhibited abnormal morphology, including dwarfism, thick stems and the formation of unviable seeds. Although the fruits of these transgenic lines accumulated two-fold higher levels of glutamate compared with non-transgenic fruits, no clear correlation was observed between *GAD-19* expression and GABA accumulation (Kisaka et al. 2006). Moreover, physiological functions of other *SlGADs* are still unclear.

To experimentally elucidate the relationship between the expression levels of each *SlGAD* gene and the accumulation of GABA in tomato fruits, here, we generated transgenic tomato plants in which each *SlGAD* or all three *SlGAD* genes were suppressed, as well as plants in which *SlGAD3* transcript was over-expressed. Furthermore, we investigated changes in primary metabolite levels in the fruits of GABA-suppressed transgenic lines to explore the physiological functions of GABA in

tomato fruits. The results suggest that both *SlGAD2* and *SlGAD3* contribute to GABA accumulation, although changes in GABA levels had little effect on tomato development or the metabolite composition in fruits.

**Results**

*Generation of SlGAD knock-down lines*

To examine the effects of *SlGAD* expression on GABA biosynthesis in tomato fruits, we generated transgenic tomato plants in which the expression of *SlGAD1, SlGAD2* or *SlGAD3* was almost specifically knocked-down (designated *RNAi-SlGAD1*, *RNAi-SlGAD2* and *RNAi-SlGAD3*, respectively) or that of all three *SlGAD*s was simultaneously knocked-down (designated *RNAi-SlGADall*) using RNAi constructs under the control of the constitutive cauliflower mosaic virus (CaMV) 35S promoter (Supplementary Fig. S1). We transformed tomato via *Agrobacterium tumefaciens*-transformation using approximately 500 cotyledon segments per construct. Regenerated plants that survived on Murashige and Skoog (MS) medium containing kanamycin were subjected to ploidy analysis, and only diploid plants were selected. We confirmed the copy number of T-DNA insertions by Southern blot analysis using a *neomycin phosphotransferase II* (*NPTII*) gene-specific probe. Eight *RNAi-SlGAD1* lines (2, 5, 6, 7, 8, 11, 13 and 14; Supplementary Fig. S2A), four *RNAi-SlGAD2* lines (3, 7, 10 and 11; Supplementary Fig. S2B), seven *RNAi-SlGAD3* lines (2, 4, 5, 6, 8, 10 and 15; Supplementary Fig. S2C) and four *RNAi-SlGADall* lines (1, 5, 7 and 8; Supplementary Fig. S2D) produced single bands, indicating that they contained single copies of T-DNA inserts in their genomes. To roughly estimate the effects of the transgenes, we measured fruit GABA levels in $T_0$ RNAi

lines using an enzymatic assay with GABase (Jakoby 1962). GABA in tomato fruits reach maximum levels at the MG stage and rapidly declined after the breaker stage (Rolin et al. 2000; Akihiro et al. 2008). Therefore, we analyzed GABA levels in fruits at the MG stage (when GABA is reported to accumulate) and at the red stage (when GABA levels are reported to decline). The GABA levels in *RNAi-SlGAD1* lines were not markedly altered compared with the WT controls (Supplementary Fig. S3A), except that line 14 had relatively high levels of GABA at the red stage (2.79-fold higher than that of WT). On the other hand, *RNAi-SlGAD2*, *RNAi-SlGAD3* and *RNAi-SlGADall* exhibited lower GABA levels compared with WT. For example, five *RNAi-SlGAD2* lines (3, 7, 8, 10 and 11) had lower GABA levels than the WT at both the MG and red stages, with 37–59% and 23–72% of WT levels, respectively (Supplementary Fig. S3B). Five *RNAi-SlGAD3* lines (3, 5, 7, 8 and 11) and four *RNAi-SlGADall* lines (1, 6, 7 and 8) also exhibited a considerable reduction in GABA levels. At the MG and red stages, the GABA levels were 5–28% and 7–36% of those of the WT, respectively (Supplementary Fig. S3C, D). These results indicate that the expression levels of *SlGAD2* and *SlGAD3* are correlated with GABA accumulation in tomato fruits. By contrast, we found no correlation between *SlGAD1* mRNA levels and GABA levels in $T_0$ *RNAi-SlGAD1* fruits (Supplementary Fig. S4), suggesting that *SlGAD1* contributes to a lesser degree to GABA accumulation in fruits. Thus, no further analysis was carried out on the *RNAi-SlGAD1* lines.

To further confirm that the reductions in fruit GABA levels in $T_0$ *RNAi-SlGAD2*, *RNAi-SlGAD3* and *RNAi-SlGADall* lines were caused by the presence of RNAi transgenes, two or three independent lines containing single copies of the T-DNA were self-pollinated, and the resulting $T_2$ or $T_3$ plants were reanalyzed in detail. To evaluate the effects of the transgenes, homozygous (HO) plants were

compared with the corresponding azygous (AZ) plants derived from the same $T_0$ plants that had lost the transgene through genetic segregation and with WT plants in all experiments. First, we performed quantitative RT-PCR analysis of *SlGADs* to determine whether the RNAi-targeted genes were effectively suppressed in the transgenic lines. In this experiment, total RNA extracted from fruits at the MG and red stages was used. The expression levels in each line were compared with those in the WT MG fruit, which were set to a baseline value of 1. The expression levels of *SlGAD2* in $T_2$ *RNAi-SlGAD2* HO fruits were 14% and 25% of WT levels in lines 3-HO and 8-HO, respectively, while AZ lines 3-AZ and 8-AZ exhibited 110% and 70% (a significantly lower level) of WT levels, respectively, at the MG stage (Fig. 1A). Although the expression of *SlGAD3* in line 8-HO was also suppressed, the expression level of *SlGAD3* in line 3-HO and the expression levels of *SlGAD1* in both HO lines showed no significant changes compared with WT (Fig. 1A). The effective suppression of *SlGAD2* was also observed in red stage fruits of the HO lines, with 3-HO and 8-HO exhibiting 32% and 22% of WT levels, respectively (Fig. 1A).

Next, we measured the GABA levels in fruits at the MG and red stages. The GABA levels were significantly reduced in the $T_2$ *RNAi-SlGAD2* HO lines. The GABA levels in lines 3-HO and 8-HO were 50% and 81% of WT levels, respectively, at the MG stage, and 25% and 61% of WT levels, respectively, at the red stage (Fig. 1D). Although the 3-AZ line also exhibited a significant reduction in GABA levels compared with WT, the levels were still higher than those of the corresponding HO line, 3-HO, at the MG and red stages. We performed the same experiments using the $T_2$ *RNAi-SlGAD3* and $T_3$ *RNAi-SlGADall* lines. For the $T_2$ *RNAi-SlGAD3* lines, the expression levels of *SlGAD3* were considerably reduced in the HO lines, with lines 5-HO and 8-HO exhibiting 6% and 4% of WT levels,

respectively, while the levels in the AZ lines were similar to those of the WT at the MG stage (Fig. 1B). The expression of *SlGAD3* in the HO lines was also significantly suppressed at the red stage, with lines 5-HO and 8-HO exhibiting 23% and 8% of WT levels, respectively (Fig. 1B). The GABA levels were positively correlated with *SlGAD3* expression levels in fruits. The GABA levels in the $T_2$ *RNAi-SlGAD3* HO lines were significantly reduced, with levels 10% and 8% of those of the WT in lines 5-HO and 8-HO, respectively, at the MG stage, and 8% and 9% of WT levels in lines 5-HO and 8-HO, respectively, at the red stage (Fig. 1E). By contrast, the GABA levels in the AZ lines were similar to those of the WT (Fig. 1E). For the $T_3$ *RNAi-SlGADall* lines, the expression levels of all three *SlGAD*s were dramatically suppressed in the HO lines compared with the WT. The expression levels of *SlGAD1*, *SlGAD2* and *SlGAD3* in the three HO lines were 2–5%, 3–5% and 5–12% of those of the WT, respectively, at the MG stage (Fig. 1C). No significant reductions were observed in any AZ line compared with the WT. Similar trends were observed in red fruit, with the expression levels of *SlGAD1*, *SlGAD2* and *SlGAD3* in the HO lines 13–25%, 5–8% and 8–23% of those of the WT, respectively (Fig. 1C). The GABA levels in HO lines 1-HO, 7-HO and 8-HO were also strongly reduced, i.e., 5%, 10% and 5% of WT levels, respectively, at the MG stage and 3%, 9% and 3% of WT levels, respectively, at the red stage (Fig. 1F).

Using MG fruits of these RNAi lines, we also determined the enzymatic activity of GAD. In *RNAi-SlGADall* lines, GAD enzyme activity in the HO lines was strongly reduced compared with WT and the corresponding AZ lines (Supplementary Fig. S5A). In *RNAi-SlGAD2*, GAD enzyme activity in the HO lines was strongly reduced, especially in 3-HO (2.7% of WT levels; Supplementary Fig. S5B). Furthermore, GAD enzyme activity in the *RNAi-SlGAD3* HO lines was significantly lower than that of

the corresponding AZ lines or WT, although these reductions were not as large as those observed in the *RNAi-SlGAD2* HO lines (Supplementary Fig. S5B, C). These results indicate that the reduced transcript levels of *SlGAD* genes result in reduced GAD enzyme activity.

Although we observed significantly lower levels of GABA in the HO fruits of the *RNAi-SlGAD2*, *RNAi-SlGAD3* and *RNAi-SlGADall* lines, we did not detect any visible abnormalities specific to the HO lines. For instance, the fruit GABA levels in $T_3$ *RNAi-SlGADall* lines (1-, 7- and 8-HO) were drastically reduced (to less than 10% of WT levels; Fig. 1F), whereas the overall plant development and fruit appearance were similar to those of the WT and the corresponding AZ lines, although some HO lines exhibited mild differences in some vegetative growth parameters (Days to flowering, Plant height or Fruit weight; Fig. 2, Supplementary Table S2). However, these changes were not consistent among the three HO lines. Additionally, we measured the levels of GABA and glutamate in the leaves of *RNAi-SlGADall* lines (Supplementary Fig. S6A). The GABA levels in all three HO lines were strongly reduced, as was the case in fruits, while no significant differences were observed in glutamate levels compared with those of the WT and AZ lines. These results suggest that the reduction in GABA levels has little effect on glutamate levels in leaves as well as tomato plant development.

*Generation of SlGAD3 over-expression lines*

Among the RNAi lines, *SlGAD3*-suppressed lines showed the strongest reduction in GABA accumulation in tomato fruits. To further evaluate the importance of *SlGAD3* expression for GABA biosynthesis in tomato fruits, we generated transgenic lines that over-expressed the full-length coding

sequence of *SlGAD3* under the control of the CaMV 35S promoter (*OX-SlGAD3*; Supplementary Fig. S7A). Tomato transformation was performed as described above, and 11 transformants were obtained. Southern blot analysis revealed that five lines (5, 6, 7, 10 and 11) harbored single copies of the T-DNA insertion (Supplementary Fig. S7B). As lines 6 and 7 had relatively high GABA levels in $T_0$ fruits at the MG and red stages, i.e., approximately 1.5- to 2-fold higher, respectively, than those of WT (data not shown), these two lines were self-pollinated and reanalyzed in detail in the $T_2$ generation. The expression levels of *SlGAD3* in two HO lines, 6-HO and 7-HO, increased more than 20-fold in MG fruits and 200-fold in red fruits compared with those of the corresponding AZ lines (6-AZ and 7-AZ) and the WT (Fig. 3A, B). As expected, the fruit GABA levels in 6-HO and 7-HO were higher as well, ranging from 2.7- to 3.3-fold and 4.0- to 5.2-fold of that of the WT at the MG and red stages, respectively (Fig. 3C, D). Increased GABA accumulation was also observed in the leaves of *OX-SlGAD3* HO lines, ranging from 14- to 17-fold WT levels, while the glutamate levels were significantly reduced, with 53–54% of WT levels (Supplementary Fig. S6B). The strong correlation between *SlGAD3* expression and GABA levels in fruits indicates that *SlGAD3* clearly functions in GABA biosynthesis in tomato fruits. However, we observed no prominent changes in visible phenotypes between the *OX-SlGAD3* HO lines and the controls (Supplementary Fig. S8; Supplementary Table S3). These results suggest that increased *SlGAD3* transcript levels lead to an increase in GABA accumulation in fruits without an associated alteration of phenotype.

*Metabolite profiling of RNAi-SlGADall lines*

To further investigate the role of SlGAD in tomato fruits, we profiled the levels of primary

metabolites, including GABA, in the fruits of three $T_3$ *RNAi-SlGADall* lines (1-, 7- and 8-HO) and compared them with those of the corresponding AZ lines (1-, 7- and 8-AZ) or the WT. In this experiment, we subjected pericarps of fruits at the MG and red stages to metabolite profiling. The primary metabolites were analyzed using gas chromatography time-of-flight mass spectrometry (GC-TOF-MS). Based on the resulting data, we analyzed only the metabolites exhibiting significantly different levels between *RNAi-SlGADall* HO lines and the corresponding AZ lines or WT. For MG fruits, when the metabolite levels in the HO lines were compared with those of the WT (HO/WT), the levels of 1, 8 and 5 metabolites were specifically reduced in the 1-, 7- and 8-HO lines, respectively, and the levels of two metabolites (unidentified metabolite MST11 and GABA) were reduced in all three HO lines (Supplementary Fig. S9A). On the other hand, the levels of 2, 12 and 8 metabolites were specifically increased in the 1-, 7- and 8-HO lines, respectively, and the levels of three metabolites (glutamate, phenylalanine and MST4) were increased in all three HO lines. Notably, different trends were observed when the metabolite levels in the HO lines were compared with those of the corresponding AZ lines (HO/AZ; Supplementary Fig. S9B). There was only one metabolite (GABA) with reduced levels in all three HO lines and none with increased levels in all three lines when compared to the AZ lines (Supplementary Fig. S9B). Additionally, the levels of only two metabolites, glutamate and alanine, increased in both lines 1- and 8-HO and lines 7- and 8-HO. No other common fluctuations in metabolite level were observed in all three HO lines when compared to the AZ lines (Supplementary Fig. S9B).

The $\log_2$ fold-changes in the levels of each metabolite, which were categorized into those that simultaneously changed in more than two of the three HO lines listed in Supplementary Fig. S9, are

shown in Fig. 4. When we compared the metabolite levels in the HO lines with those of WT (HO/WT), we determined that the levels of 15 metabolites were significantly altered (Fig. 4A). The most considerable changes were observed in GABA levels, which were reduced in all three HO lines, which is consistent with the results obtained by the GABase assay (Fig. 1F). Among the metabolites with increased levels, the levels of glutamate (a precursor to GABA), phenylalanine and an unidentified metabolite, MST4, were significantly altered in all three HO lines (Fig. 4A). By contrast, when the metabolite levels in the HO lines were compared with those in the corresponding AZ lines (HO/AZ), the levels of only three metabolites were significantly altered (Fig. 4B). As observed in the comparison with WT, the GABA levels were considerably reduced in all three HO lines. On the other hand, the levels of glutamate and alanine increased in two of the three HO lines (Fig. 4B). Only the accumulation patterns of GABA and glutamate exhibited similar tendencies in a comparison between HO/WT and HO/AZ data at the MG stage (Fig. 4A, B). The same experiment was conducted with red stage fruits. When the metabolite levels in the HO lines were compared with those of WT (HO/WT), the levels of two metabolites were reduced in lines 1- and 7-HO and in all three HO lines (Supplementary Fig. S10A). On the other hand, the levels of 17, 1 and 11 metabolites were commonly increased in the 1- and 7-HO lines, the 1- and 8-HO lines and all three HO lines, respectively (Supplementary Fig. S10A). As observed in MG fruits, when the metabolite levels in the HO lines were compared with those of the corresponding AZ lines (HO/AZ), the levels of very few metabolites were altered in all three HO lines, and the levels of only four metabolites were significantly altered in more than two of the three HO lines (Supplementary Fig. S10B). The $\log_2$ fold-changes in the levels of these commonly altered metabolites are shown in Fig. 5. A prominent reduction in GABA levels was also observed in the three HO fruits at

the red stage compared with both the WT and the corresponding AZ lines. Only GABA levels were consistently different between HO/WT and HO/AZ, although the levels of many metabolites were significantly different between all three HO lines and the WT (Fig. 5A, B).

**Discussion**

*SlGAD2 and SlGAD3 appear to be major isoforms regulating GABA production/accumulation in tomato fruits*

In various organisms, including higher plants, GAD is considered to be the key enzyme in GABA biosynthesis (Akbarian and Huang 2006; Bouché and Fromm 2004a; Komatsuzaki et al. 2008). We previously isolated three *GAD* gene homologs (*SlGAD1*, *SlGAD2* and *SlGAD3*) from tomato cv. 'Micro-Tom' fruits and found that the increase in GABA levels during fruit development is correlated with the expression of *SlGAD2* and *SlGAD3* (Akihiro et al. 2008). In this study, to investigate whether *SlGADs* are indeed involved in GABA biosynthesis in tomato fruits, we generated transgenic tomato plants in which each *SlGAD*, or all three *SlGAD*s, were suppressed by RNAi technology. The fruit GABA levels in the $T_3$ *RNAi-SlGADall* lines exhibited significant reductions (to less than 10% of WT levels; Fig. 1F), which were associated with the effective suppression of the three *SlGAD*s and reduced enzyme activity (Fig. 1C, Supplementary Fig. S5A). This result suggests that the main route of GABA biosynthesis in tomato fruits is through the decarboxylation of glutamate by GAD enzymes. Reduced levels in fruit GABA were also observed in the *RNAi-SlGAD2* and *RNAi-SlGAD3* lines. In particular, *SlGAD3* suppression had a greater impact on fruit GABA levels, which were reduced to the same extent as in the *RNAi-SlGADall* lines (Fig. 1E, F), while *SlGAD2* suppression also led to

somewhat lower GABA levels, with 50–81% and 25–61% of WT levels detected in MG and red fruits, respectively (Fig. 1D). These results suggest that both *SlGAD2* and *SlGAD3* function in GABA biosynthesis in tomato fruits. However, although GAD enzyme activity was more efficiently suppressed in the *RNAi-SlGAD2* HO lines than in the *RNAi-SlGAD3* HO lines (Supplementary Fig. S5B, C), total GABA levels exhibited greater reductions in the *RNAi-SlGAD3* HO lines (Fig. 1D, E). These results suggest that GABA levels in fruits are more closely correlated with the mRNA levels of *SlGAD* genes than with their enzyme activities.

To further explore the function of *SlGAD* genes, we created *OX-SlGAD3* lines. Fluctuations in GABA levels associated with the modulation of *SlGAD3* expression were also observed in the *OX-SlGAD3* lines, in which fruit GABA levels increased to 2.7- to 5.2-fold that of WT at the MG and red stages, respectively (Fig. 3C, D), supporting the notion that *SlGAD3* functions in GABA biosynthesis in tomato fruits. By contrast, fruit GABA levels in the $T_0$ *RNAi-SlGAD1* lines were similar to those of the WT (Supplementary Fig. S3A), suggesting that the contribution of *SlGAD1* to general GABA biosynthesis in tomato fruits may not be as important as those of the other two *SlGAD* genes.

*Varied GABA levels do not affect tomato fruit properties or vegetative growth*

Although little is known about the effects of reduced GABA levels on plant cells, it is well known that excessive levels of GABA cause severe abnormalities in plant vegetative and reproductive development. For instance, transgenic tobacco and rice plants expressing mutated GAD in which the C-terminal extension domains were removed accumulated higher levels of GABA in stems and leaves than WT and exhibited dwarfism, etiolated leaves and sterility (Baum et al. 1996; Akama and Takaiwa

2007). The *Arabidopsis* GABA-T-deficient mutant *pop2* is defective in pollen tube growth and cell elongation in primary roots and hypocotyls when exposed to high concentrations of exogenous GABA (Palanivelu et al. 2003; Renault et al. 2011). Furthermore, transgenic tomato plants in which tomato *GABA-T* (*SlGABA-T*) genes are suppressed exhibit severe dwarfism and infertility (Koike et al. 2013). These findings indicate that GABA affects certain types of physiological responses in plants. When we compared the vegetative growth and fruit development of the *RNAi-SlGADall* lines with those of the WT, some HO lines exhibited significant differences, but these altered phenotypes were not consistently found among the three HO lines (Supplementary Table S2). These results indicate that the observed differences were not due to the reductions in GABA levels. In addition, most parameters (especially plant height and number of leaves) were similar between HO lines and the corresponding AZ lines, indicating that the differences between HO lines and the WT were likely due to the somaclonal variations often observed in clonally propagated plants (Miguel and Marum 2011) and not to reduced GABA levels. Additionally, the increased levels of GABA in fruits and leaves of *OX-SlGAD3* did not result in any visible abnormalities in fruits or vegetative organs (Supplementary Fig. S8; Supplementary Table S3). Therefore, our findings do not support the results of previous studies. The effects of differences in GABA over-accumulation may arise from differences in the capacity for GABA accumulation. Under non-stress conditions, the GABA levels in vegetative tissues are generally low (Satya-Narayan and Nair 1990). However, large fluctuations in GABA levels occur naturally during tomato fruit development, suggesting that tomato fruit tissue may have a strong capacity to store GABA. Recently, Snowden et al. (2015) hypothesized that in the highly vacuolated pericarp cells, GABA is mainly stored in the vacuole. The authors demonstrated the existence of a tonoplast

Glu/Asp/GABA exchanger (SlCAT9) and revealed that SlCAT9 strongly affects the accumulation of GABA, Glu and Asp during tomato fruit development (Snowden et al. 2015). These findings suggest that the capacity to transport GABA across the vacuole and the differences in compartmentalization may affect GABA accumulation and the sensitivity to excessive levels of GABA in plant cells.

Alternatively, it is possible that plant morphology is susceptible not only to excessive GABA accumulation itself, but also to some other factors that arise from defects in GABA catabolism. For example, *Arabidopsis ssadh*-deficient mutants exhibit more severe dwarfism and necrotic lesions than WT (Bouché et al. 2003). Since the severe phenotype observed in the *ssadh* single mutant is suppressed in the *gaba-t ssadh* double mutant, the phenotype is considered to be due to the accumulation of γ-hydroxybutyrate, which is produced downstream of the GABA-T transamination step (Ludewig et al. 2008). However, transgenic tomato plants in which *SlGABA-T* genes are suppressed also exhibit severe dwarfism and infertility (Koike et al. 2013). Thus, other factors impairing normal plant development might exist in tomato.

Although the effects of excessive levels of GABA in tomato plants and fruits were not observed in the current study, we did find that the down-regulation of GABA biosynthesis had little effect on tomato plant growth and fruit development under normal growth conditions.

*Why do GABA levels change during fruit development?*

GABA is the predominant free amino acid in green stage fruits (Boggio et al. 2000; Sorrequieta et al. 2010), and in a cherry tomato cultivar, its levels reach 50% of total free amino acid contents at the MG stage (Rolin et al. 2000). However, such a large amount of GABA must be

catabolized rapidly during the ripening stage. To further explore the reason for this dynamic change in GABA levels during tomato fruit development, we performed metabolite profiling using *RNAi-SlGADall* fruits and investigated the effects of reduced GABA levels on the levels of other metabolites. In this experiment, we compared the levels of each primary metabolite in *RNAi-SlGADall* lines 1-, 7- and 8-HO with those of the WT or the corresponding AZ lines 1-, 7- and 8-AZ. Since both the WT and AZ lines have normal GABA levels (Fig. 1F), if the reduced GABA levels affect the levels of other metabolites in the HO lines, the trends in metabolite levels should be consistently observed between the HO/WT and HO/AZ data. However, large differences were detected between the numbers of metabolites that exhibited significant changes in the HO/WT versus the HO/AZ data (Figs. 4, 5). In MG fruits, for example, when each metabolite level was compared with that of WT (HO/WT), 15 metabolites exhibited significant differences in more than two of the three HO lines (Fig. 4A). However, when each metabolite level was compared with that of the corresponding AZ lines (HO/AZ), only three metabolites exhibited significant differences in two of the three HO lines (Fig. 4B). Among these detected metabolites, only GABA and glutamate exhibited common trends in both the HO/WT and HO/AZ data (Fig. 4A, B). The changes in the third metabolite, alanine, were not consistent among the three HO lines when compared with the AZ lines (Fig. 4B). These results suggest that reduced GABA levels only affect glutamate levels in the MG fruits of *RNAi-SlGADall* lines. In red fruits, although the levels of 33 metabolites exhibited significant differences between HO lines and WT (HO/WT; Fig. 5A), the levels of only four metabolites exhibited significant differences between HO lines and AZ lines (HO/AZ; Fig. 5B). Surprisingly, there was no common metabolite, other than GABA, that fluctuated in both the HO/WT and HO/AZ data in red fruits (Fig. 5A, B).

In general, variations caused by transgenes or tissue culture can be assessed separately by comparing transgenic lines and their AZ lines, or WT and AZ lines, respectively (Zhou et al. 2012). In the present study, the number of metabolites with significantly altered levels was much lower in HO/AZ than in HO/WT, suggesting that the somaclonal variations caused by tissue culture may also have a major effect on metabolite levels in transgenic plants. Kusano et al. (2011) reported that >92% of the fruit metabolites detected in a transgenic tomato over-expressing miraculin, a taste-modifying protein, exhibited fewer fluctuations than those observed among non-transgenic traditional cultivars. The authors performed consensus orthogonal partial least squares discriminant analysis to detect the changes in metabolite levels attributable to the genetic modification. Although they did not perform a comparative analysis of transgenic versus AZ lines, the authors also suggested that heritable epigenetic regulations could affect the metabolite changes in the transgenic lines. Zhou et al. (2012) evaluated the potential metabolic variations caused by gene insertion and tissue culture using AZ lines. In this study, the metabolic variations in a transgenic rice line harboring the *Bacillus thuringiensis δ-endotoxin* and *cowpea trypsin inhibitor* genes were evaluated by GC-MS-based metabolic profiling, principal component analyses and statistical analyses. The results showed that the metabolic variations between transgenic lines and null-segregants (AZ lines) were smaller than those between null-segregants (AZ lines) and WT, suggesting that a large proportion of the observed metabolic changes were attributed to the effects of tissue culture.

The up-regulation of GAD in plants strongly affects the levels of other metabolites. For example, in rice calli over-expressing the C-terminal truncated version of *OsGAD2*, which leads to elevated GABA levels, the amounts of many amino acids (such as glutamate, aspartate, threonine,

asparagine and glutamine) are reduced (Akama and Takaiwa et al. 2007). In *Arabidopsis* seeds over-expressing the C-terminal truncated version of petunia *GAD*, a major alteration in the C–N balance during seed maturation was observed (Fait et al. 2011). By contrast, in the present study, we found that down-regulating *GAD* gene expression had little effect on the levels of other metabolites in tomato fruits (Figs. 4, 5). The increase in (only) glutamate levels observed in MG fruits may be a consequence of the efficient suppression of *SlGADs*, because glutamate is a direct precursor of GABA and is used as a substrate for GAD enzymes. However, the change in glutamate levels was not as great as the reduction in GABA levels (Fig. 4). Similarly, the roots of *Arabidopsis* GAD1-deficient mutants (Bouché et al. 2004b) exhibit considerable reductions in GABA levels (7-fold lower than WT) with no significant changes in glutamate levels. Glutamate also plays a central role in plant nitrogen metabolism and acts not only as a precursor for GABA, proline, arginine (which itself acts as a precursor of polyamines) and 2-oxoglutarate, but also as a substrate for a wide range of aminotransferase reactions forming various amino acids, such as aspartate, alanine and glycine (Forde and Lea 2007). Therefore, some of the excessive glutamate that was not used by SlGADs may have been metabolized to other compounds by a large number of different enzymes involved in glutamate metabolism, and thus glutamate levels did not increase greatly in MG fruits of the *RNAi-SlGADall* lines. In red fruits, even glutamate levels were not significantly altered in the *RNAi-SlGADall* lines (Fig. 5). In some tomato cultivars including ‘Micro-Tom’, high GAD activity has been observed in green fruits but not in red fruits (Rolin et al. 2000; Boggio et al. 2000; Akihiro et al. 2008), which may explain why glutamate levels were not affected by *SlGAD* suppression in red fruits in the *RNAi-SlGADall* lines.

In conclusion, the present study experimentally demonstrates that the expression of *SlGAD2* and *SlGAD3* is crucial for GABA biosynthesis in tomato fruits. This information will be useful for further studies aimed at developing a tomato that hyper-accumulates GABA. Although we did not uncover a new function for GABA in tomato fruits, we determined that the down-regulation of GABA biosynthesis has little effect on plant growth, fruit development or fruit primary metabolism under normal growth conditions. Therefore, we conclude that GABA accumulation may not be a critical factor for normal fruit development, at least under normal growth conditions.

**Materials and Methods**

*Vector construction and tomato transformation*

To generate *SlGAD* knock-down lines, RNAi constructs were prepared using the Gateway system (Invitrogen). Since the coding sequences of the three *SlGADs* share a relatively high homology (68–77% nucleotide identity), partial cDNA fragments of approximately 300 bp at the 3′ ends of the coding sequences and 3′ UTRs, where the homology among three *SlGAD* cDNA sequences is relatively low (26–48% nucleotide identity), were amplified by PCR as the RNAi-targeted regions of *RNAi-SlGAD1*, *RNAi-SlGAD2* and *RNAi-SlGAD3* (Supplementary Fig. S1). By contrast, the RNAi-targeted region of *RNAi-SlGADall* was amplified from an approximately 150 bp portion of the coding sequence of *SlGAD2*, which corresponds to the highly conserved region sharing >76% nucleotide identity among the three *SlGAD* cDNA sequences (Supplementary Fig. S1). Primers used in this experiment are shown in Supplementary Table S1. These resulting fragments were cloned into the entry vector pCR8/GW/TOPO (Invitrogen) and sub-cloned into the pANDA35HK vector (Miki and

Shimamoto 2004) or the pBI-sense, antisense GW vector (Inplanta Innovations) using the Gateway LR Clonase enzyme (Invitrogen). Both pANDA35HK and the pBI-sense, antisense GW vectors are binary vectors that express short hairpin RNAs derived from a given gene under the control of the constitutive CaMV 35S promoter. RNAi constructs targeting *SlGAD1*, *SlGAD2* and *SlGAD3* independently, and all three *SlGAD* genes simultaneously, were designated *RNAi-SlGAD1*, *RNAi-SlGAD2*, *RNAi-SlGAD3* and *RNAi-SlGADall*, respectively.

For the *SlGAD3* gene, an over-expression construct was also created under the control of the CaMV 35S promoter. The coding sequence of *SlGAD3* was amplified by PCR using gene-specific primers (Supplementary Table S1). The PCR fragment (1,455 bp) was cloned into entry vector pCR8/GW/TOPO (Invitrogen) and sub-cloned into pBI-OX-GW (Inplanta Innovations) using the Gateway LR Clonase enzyme (Invitrogen). This construct was designated *OX-SlGAD3*.

These constructs were then transformed into *A. tumefaciens* strain GV2260 via electroporation. Cotyledons of tomato cv. 'Micro-Tom' were prepared for transformation by *Agrobacterium* harboring each construct. Transformation into tomato was performed according to the highly efficient protocol established by Sun et al. (2006). Only diploid plants were selected from among the regenerated plants that survived on MS plates containing kanamycin (100 mg $l^{-1}$). Selected plants were then transferred to Rockwool cubes (5 × 5 × 5 cm) and placed in a growth room maintained at 25°C under a 16 h light/8 h dark photoperiod of fluorescent light. Plants were supplied with a standard nutrient solution (Otsuka A; Otsuka Chemical Co., Ltd., Osaka Japan).

*Southern blot analysis*

To determine the transgene copy number in transgenic tomatoes, genomic DNA was extracted from 0.2 g fresh leaves using Maxwell 16 DNA purification kits according to the manufacturer's protocol (Promega). Extracted genomic DNA (10 μg) was digested with *Eco*RI, electrophoresed on a 0.8% agarose gel at 50 V for 3 h and transferred to a Hybond-N+ nylon membrane (GE Healthcare). The membrane was hybridized overnight at 60°C in high-SDS buffer (50% deionized formamide [v/v], 5× SSC, 7% SDS, 2% Blocking Reagent [Roche Diagnostics], 50 mM sodium phosphate [pH 7.0] and 0.1% N-lauroylsarcosine sodium salt [w/v]) containing an *NPTII* gene-specific DIG-labeled probe at 45°C. The *NPTII*-specific probe was prepared with a PCR DIG Probe Synthesis Kit (Roche) according to the manufacturer's protocol. The primer pairs used for probe synthesis are shown in Supplementary Table S1. The hybridization signals were detected using an LAS 4000 mini Image Analyzer (Fujifilm Co. Ltd.).

*Plant selection and plant growth conditions*

Two or three independent transgenic lines containing single copies of T-DNA inserts were selected from the $T_0$ generation of *RNAi-SlGAD2*, *RNAi-SlGAD3*, *RNAi-SlGADall* and *OX-SlGAD3*. In their $T_1$ generations, homozygous plants were selected by genomic real-time PCR as described by Hirai et al. (2011). Azygous plants were also selected by genomic PCR as the controls for homozygous plants.

$T_1$–$T_3$ transgenic and control plants (WT and azygous) were cultivated as follows: tomato seeds were germinated on moistened filter paper and transferred to Rockwool cubes (5 × 5 × 5 cm). Plants were grown in a closed cultivation system (Naeterasu), which was developed commercially by

Taiyo Kogyo Co., Ltd. (Tokyo, Japan) as described by Hirai et al. (2010). Plants were supplied with a standard nutrient solution (Otsuka A; Otsuka Chemical Co., Ltd., Osaka Japan).

*RNA extraction and qRT-PCR analysis*

To determine the expression levels of *SlGAD* genes in tomato fruits, qRT-PCR was performed. For RNA extraction, tomato fruits harvested at the MG stage (26–28 days after flowering) and the red stage (44–46 days after flowering) were frozen and ground to a fine powder in liquid nitrogen using a mortar and pestle. These samples were also used for GABA measurements. Total RNA was extracted using an RNeasy Plant Minikit (Qiagen) with RNase-free DNase (Qiagen). First-strand cDNA was synthesized from 1 μg of total RNA using a SuperScript VILO cDNA Synthesis Kit (Invitrogen). The cDNA was diluted 10-fold with RNase-free water and used as a template for qRT-PCR. The qRT-PCR was performed using a Thermal Cycler Dice Real Time System TP800 (Takara-Bio Inc.) with SYBR Premix Ex Taq II (Takara-Bio Inc.). The reaction cycles were as follows: 95°C for 10 s for initial denaturation, followed by 40 cycles of 5 s of denaturation at 95°C, and 30 s of annealing/extension at 54°C. Relative quantification of each *SlGAD* gene expression level was normalized to the expression level of the *Slubiquitin3* gene (GenBank accession number X58253), which was used as an internal control. The primer sequences used in this experiment are shown in Supplementary Table S1.

*Measurements of GABA and amino acid contents in fruits*

To extract free amino acids, 50 mg of powdered fruits was homogenized in 500 μl of 8%

(w/v) trichloroacetic acid and centrifuged at 10,000 × *g* for 20 min at 4°C. The supernatant was transferred to a new tube and mixed vigorously with 400 µl of pure diethyl ether for 10 min. The solution was centrifuged at 10,000 × *g* for 10 min at 4°C. The upper phase (diethyl ether) was removed and the lower phase was again mixed vigorously with 400 µl of pure diethyl ether for 10 min. After centrifugation at 10,000 × *g* for 10 min at 4°C, the upper phase was removed and the tubes were left under a draft for 1 h to evaporate the remaining diethyl ether completely. The GABA content was measured using the GABase assay as described by Jakoby (1962) with slight modifications.

For GABA and glutamate determination in leaves, amino acids were extracted from leaves as described above. Purification of the samples and amino acid analysis were performed as described by Koike et al. (2013).

*Enzyme extraction and determination of GAD activity*

MG fruits were harvested and immediately frozen in liquid nitrogen after removing seeds and jelly tissues. Five frozen fruits were ground to a fine powder in liquid nitrogen using a mortar and pestle. To extract crude protein, powdered fruits were homogenized in 5-fold volumes of ice-cold extraction buffer containing 50 mM TRIS-HCl buffer (pH 8.2), 3 mM dithiothreitol, 1.25 mM EDTA, 2.5 mM $MgCl_2$, 10% 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 et al. (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 *bis-tris*-HCl (pH 7.0), 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.

*Metabolite profiling*

Fruit samples at the MG and red stages in the $T_3$ generation were harvested from five or six independent plants per genotype to evaluate biologically oriented fluctuations in the metabolite profile data. Pericarp tissues were sampled and immediately frozen in liquid nitrogen. For GC-TOF-MS analysis, samples were lyophilized. A total of 2.5 mg dry weight (DW) of the samples was subjected to derivatization and an equivalent of 2.8 µg DW of the derivatized samples was analyzed by Leco Pegasus IV GC-TOFMS (Leco, St. Joseph, MI, USA). Data processing and normalization were performed as described in Kusano et al. (2011).

*Statistical analysis*

All statistical analyses were performed by two-tailed Student's *t*-test (Microsoft Excel) and statistical significance was evaluated at $P < 0.05$ or $P < 0.01$.

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**Disclosures**

Conflicts of interest: No conflicts of interest declared.

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**Figure legends**

**Fig. 1**

Effects of *SlGAD* suppression on the fruit GABA levels in RNAi lines. Relative expression levels of *SlGAD1*, *SlGAD2* and *SlGAD3* in the MG and red fruits of $T_2$ *RNAi-SlGAD2* (A), $T_2$ *RNAi-SlGAD3* (B), and $T_3$ *RNAi-SlGADall* (C) lines were determined by qRT-PCR. *Slubiquitin3* gene was used for normalization. GABA contents in the same sample used for qRT-PCR were also determined (D, E, F). All results are the mean±SE (n=4). Asterisks indicate a significant difference between WT and HO, or

WT and AZ according to Student's *t*-test (**P*<0.05, ***P*<0.01). MG, mature green; WT, wild-type; HO, homozygous; AZ, azygous; FW, fresh weight.

**Fig. 2**

Plant and fruit phenotypes of $T_3$ *RNAi-SlGADall.* Three independent homozygous lines of *RNAi-SlGADall* (1-, 7- and 8-HO) were grown with the corresponding azygous lines (1-, 7- and 8-AZ) and WT. Their phenotypes of whole plants at 30, 60 and 90 DAS and fruits at 45 DAF are shown. DAS, days after sowing; DAF, days after flowering.

**Fig. 3**

qRT-PCR analysis of *SlGAD* genes and GABA content in $T_2$ *OX-SlGAD3* fruits. Two independent homozygous lines of *OX-SlGAD3* (6- and 7-HO), the corresponding azygous lines (6-, and 7-AZ) and WT were analyzed. Relative expression levels of *SlGAD1, SlGAD2* and *SlGAD3* in fruits at the MG (A) and red (B) stages were determined by qRT-PCR. *Slubiquitin3* gene was used for normalization. The GABA content in fruits at the MG (C) and red (D) stages were determined. All results are the mean±SE (n=4). Asterisks indicate a significant difference between WT and HO, or WT and AZ according to Student's *t*-test (**P*<0.05, ***P*<0.01). MG, mature green; WT, wild-type; HO, homozygous; AZ, azygous; FW, fresh weight.

**Fig. 4**

Metabolites that show common significant changes in the MG fruits of more than two of the three

independent *RNAi-SlGADall* homozygous lines (1-, 7- and 8-HO) compared with WT or the corresponding azygous lines (1-, 7- and 8-AZ). HO/WT (A) or HO/AZ (B) ratio of lines 1, 7 and 8 are shown on a $\log_2$ scale. The mean values of six replicates are shown. Asterisks indicate a significant difference between HO and WT, or HO and AZ according to *t*-test (**P*<0.05, ***P*<0.01). MST, mass spectral tag (unidentified metabolite).

**Fig. 5**

Metabolites that show common significant changes in the red fruits of more than two of the three independent *RNAi-SlGADall* homozygous lines (1-, 7- and 8-HO) compared with WT or the corresponding azygous lines (1-, 7- and 8-AZ). HO/WT (A) or HO/AZ (B) ratio of lines 1, 7 and 8 are shown on a $\log_2$ scale. The mean values of six replicates are shown. Asterisks indicate a significant difference between HO and WT, or HO and AZ according to *t*-test (**P*<0.05, ***P*<0.01). MST, mass spectral tag (unidentified metabolite).

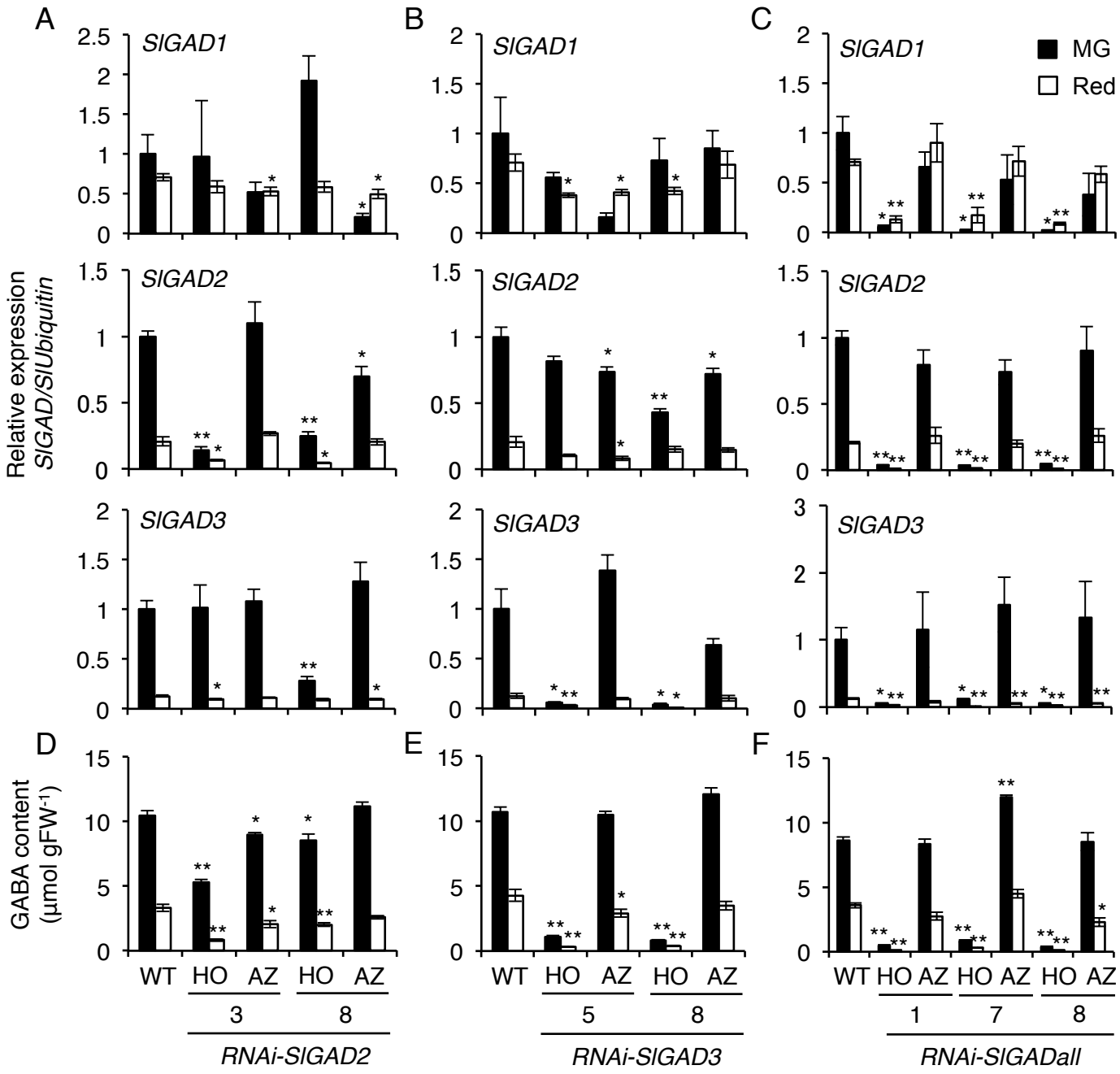



**Fig. 1**
Effects of *SlGAD* suppression on the fruit GABA levels in RNAi lines. Relative expression levels of *SlGAD1*, *SlGAD2* and *SlGAD3* in the MG and red fruits of $T_2$ *RNAi-SlGAD2* (A), $T_2$ *RNAi-SlGAD3* (B), and $T_3$ *RNAi-SlGADall* (C) lines were determined by qRT-PCR. *Slubiquitin3* gene was used for normalization. GABA contents in the same sample used for qRT-PCR were also determined (D, E, F). All results are the mean±SE (n=4). Asterisks indicate a significant difference between WT and HO, or WT and AZ according to Student's *t*-test (**P*<0.05, ***P*<0.01). MG, mature green; WT, wild-type; HO, homozygous; AZ, azygous; FW, fresh weight.

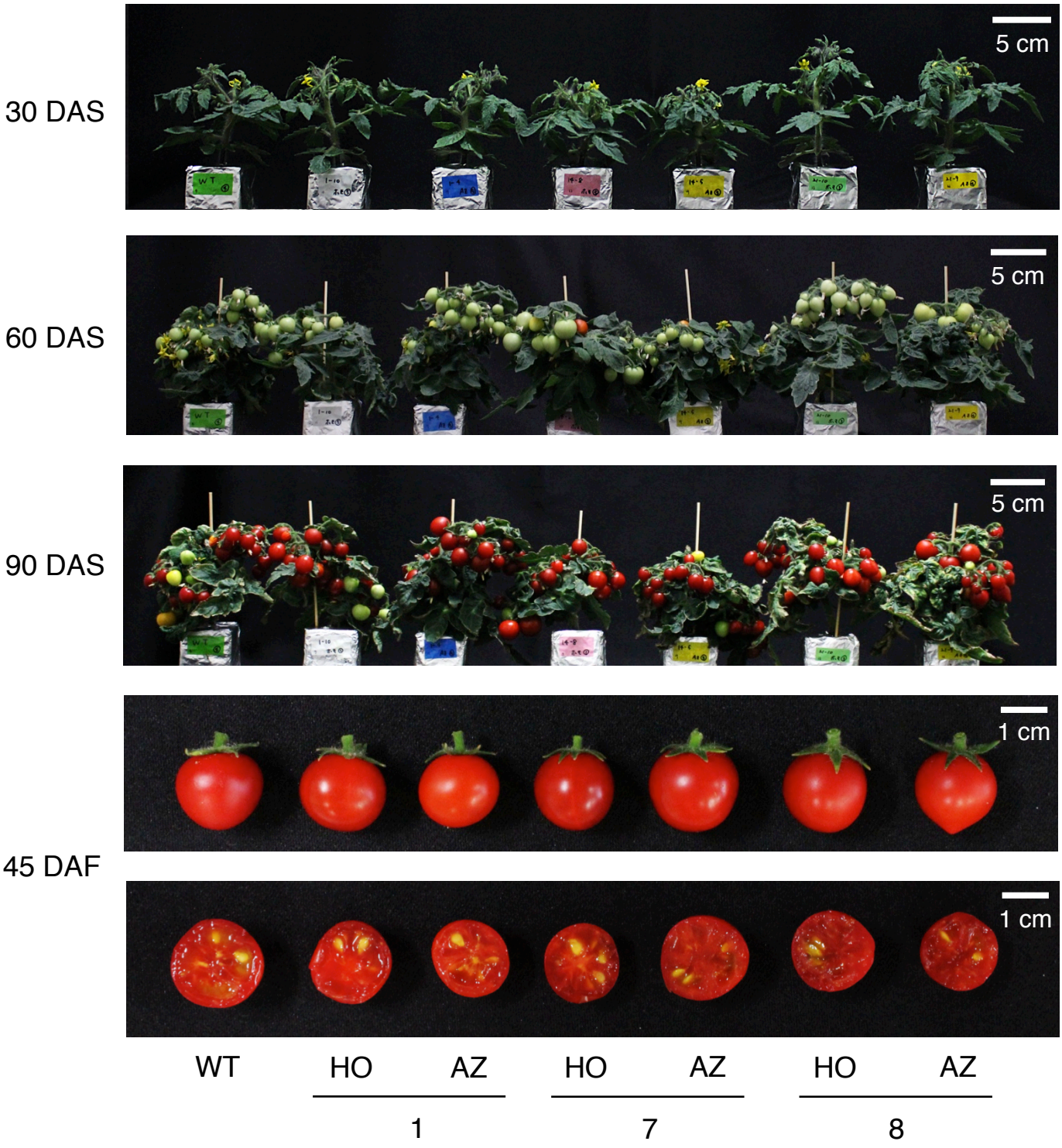



**Fig. 2**
Plant and fruit phenotypes of $T_3$ *RNAi-SlGADall.* Three independent homozygous lines of *RNAi-SlGADall* (1-, 7- and 8-HO) were grown with the corresponding azygous lines (1-, 7- and 8-AZ) and WT. Their phenotypes of whole plants at 30, 60 and 90 DAS and fruits at 45 DAF are shown. DAS, days after sowing; DAF, days after flowering.

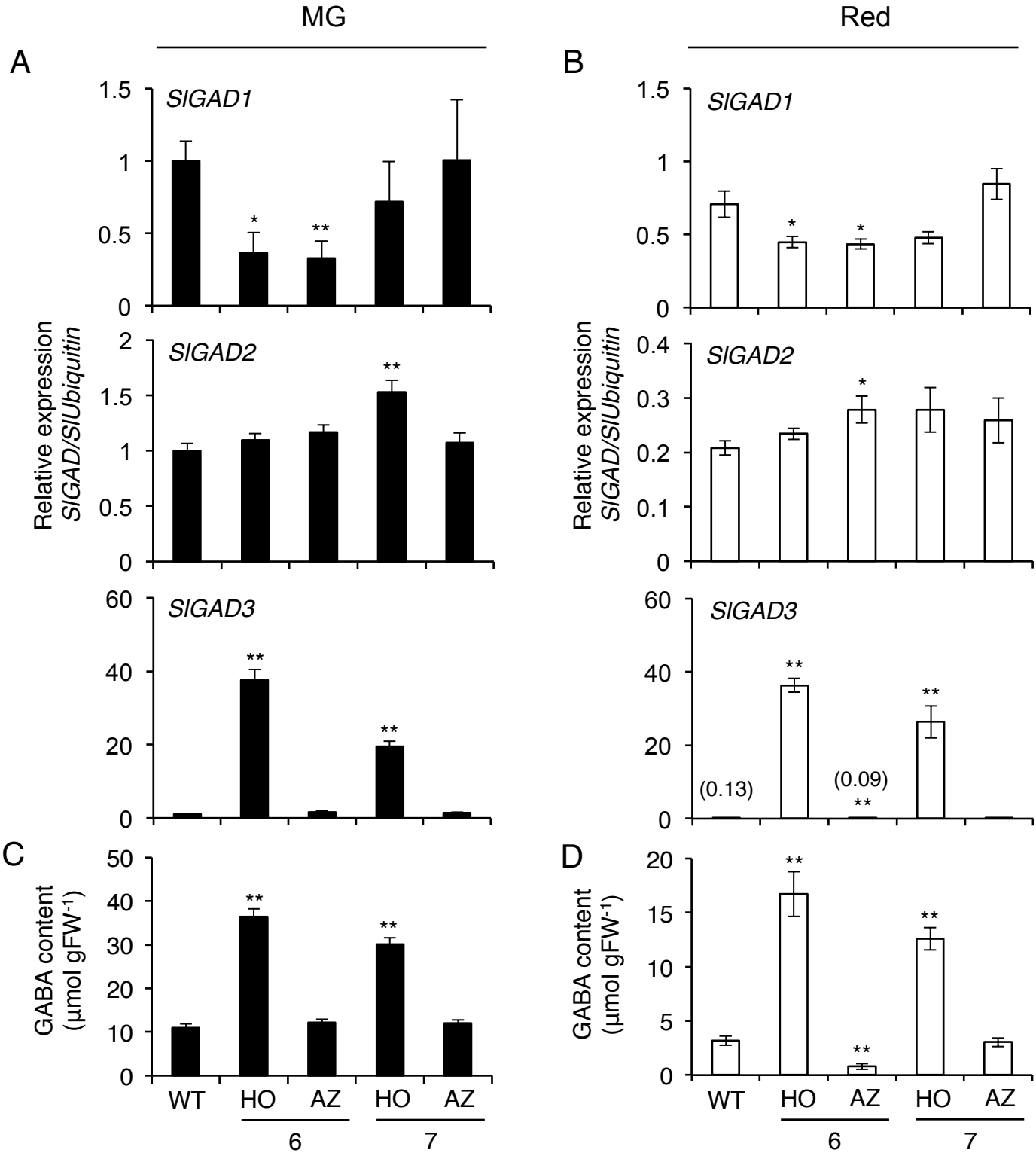



**Fig. 3**
qRT-PCR analysis of *SlGAD* genes and GABA content in $T_2$ *OX-SlGAD3* fruits. Two independent homozygous lines of *OX-SlGAD3* (6- and 7-HO), the corresponding azygous lines (6-, and 7-AZ) and WT were analyzed. Relative expression levels of *SlGAD1, SlGAD2* and *SlGAD3* in fruits at the MG (A) and red (B) stages were determined by qRT-PCR. *Slubiquitin3* gene was used for normalization. The GABA content in fruits at the MG (C) and red (D) stages were determined. All results are the mean±SE (n=4). Asterisks indicate a significant difference between WT and HO, or WT and AZ according to Student's *t*-test (**P*<0.05, ***P*<0.01). MG, mature green; WT, wild-type; HO, homozygous; AZ, azygous; FW, fresh weight.

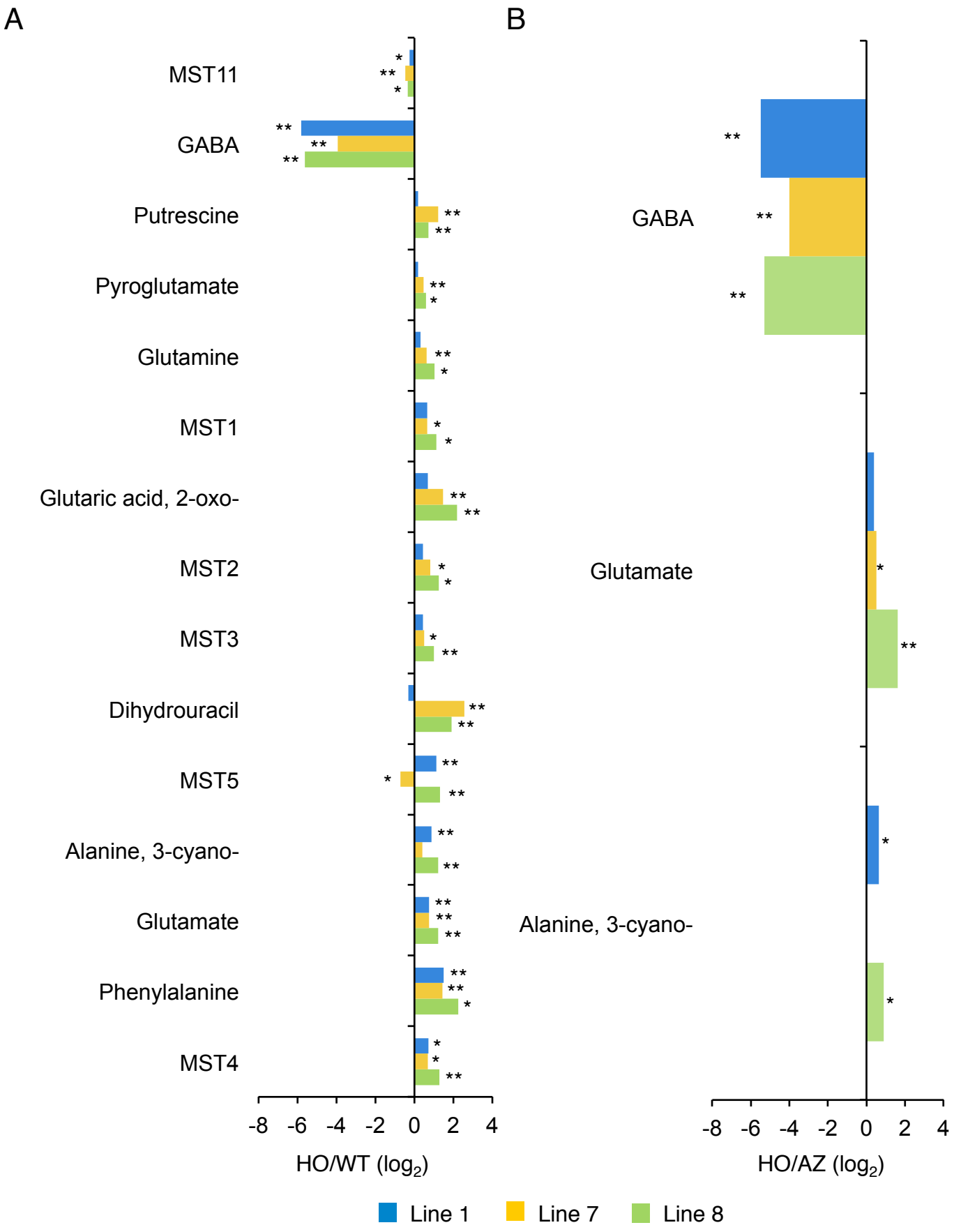



**Fig. 4**
Metabolites that show common significant changes in the MG fruits of more than two of the three independent *RNAi-SlGADall* homozygous lines (1-, 7- and 8-HO) compared with WT or the corresponding azygous lines (1-, 7- and 8-AZ). HO/WT (A) or HO/AZ (B) ratio of lines 1, 7 and 8 are shown on a $\log_2$ scale. The mean values of six replicates are shown. Asterisks indicate a significant difference between HO and WT, or HO and AZ according to *t*-test (*$P$<0.05, **$P$<0.01). MST, mass spectral tag (unidentified metabolite).

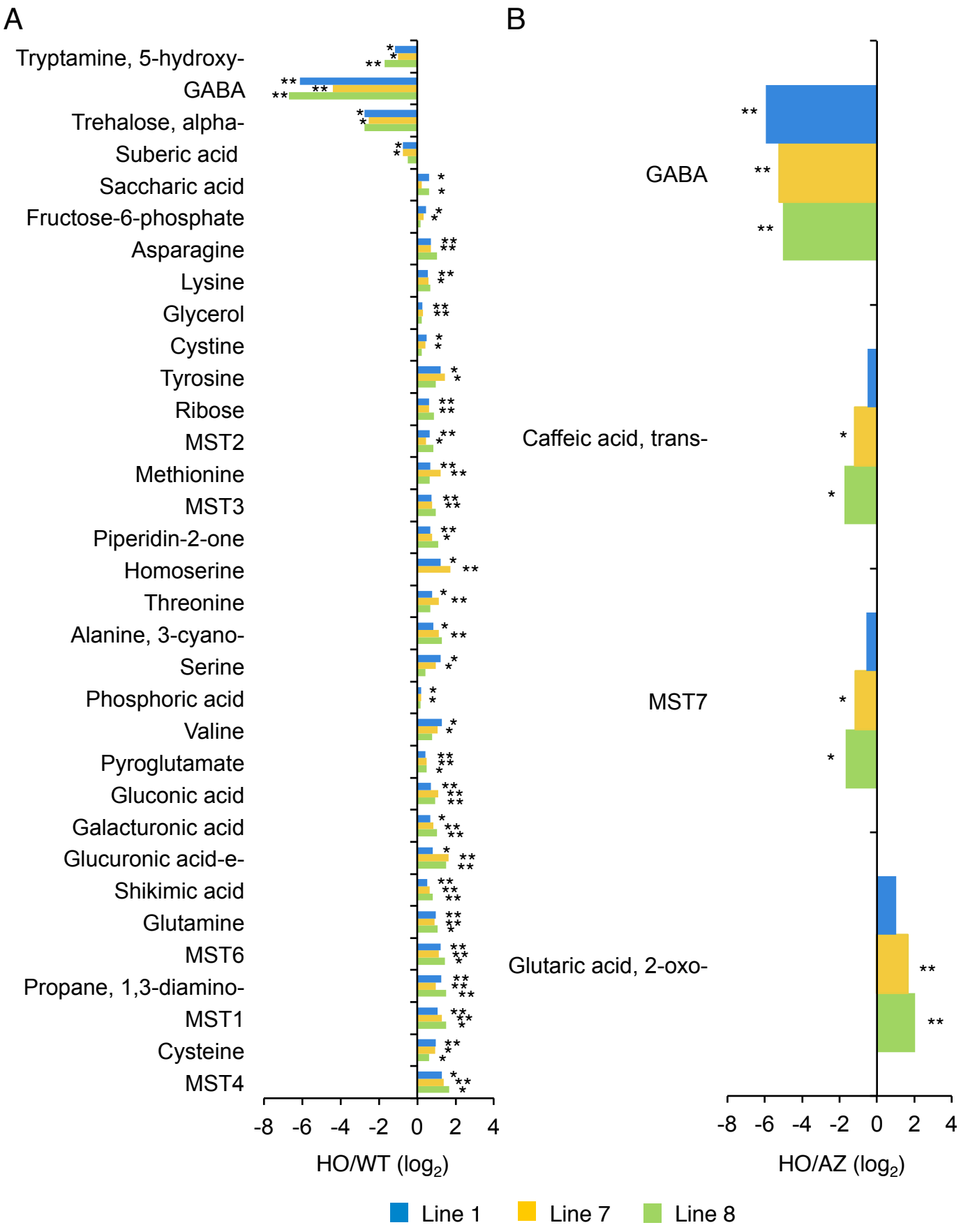


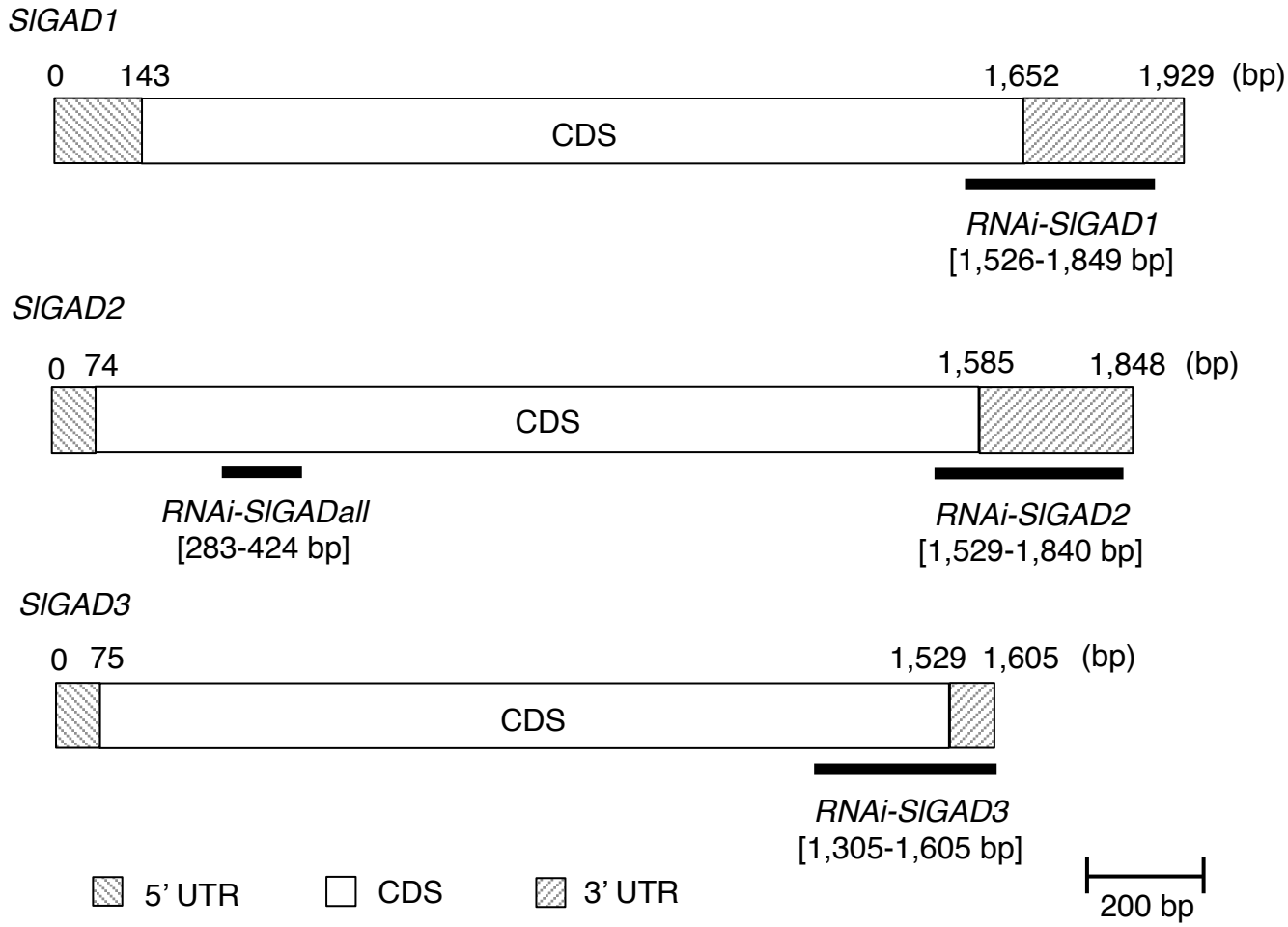

**Fig. 5**
Metabolites that show common significant changes in the red fruits of more than two of the three independent *RNAi-SlGADall* homozygous lines (1-, 7- and 8-HO) compared with WT or the corresponding azygous lines (1-, 7- and 8-AZ). HO/WT (A) or HO/AZ (B) ratio of lines 1, 7 and 8 are shown on a $\log_2$ scale. The mean values of six replicates are shown. Asterisks indicate a significant difference between HO and WT, or HO and AZ according to *t*-test (**P*<0.05, ***P*<0.01). MST, mass spectral tag (unidentified metabolite).

# Supplementary Data

Tomato Glutamate Decarboxylase Genes *SlGAD2* and *SlGAD3*
Play Key Roles in Regulating γ-Aminobutyric Acid Levels in Tomato
(*Solanum lycopersicum*)

Mariko Takayama, Satoshi Koike, Miyako Kusano,
Chiaki Matsukura, Kazuki Saito, Tohru Ariizumi,
Hiroshi Ezura

A




B

| RNAi-targeted regions | *SlGAD1* | *SlGAD2* | *SlGAD3* |
|---|---|---|---|
| *RNAi-SlGAD1* | 100.0 % (324 nt) | 47.6 % ( 6 nt) | 34.6 % ( 7 nt) |
| *RNAi-SlGAD2* | 47.1 % ( 6 nt) | 100.0 % (312 nt) | 26.1 % ( 8 nt) |
| *RNAi-SlGAD3* | 42.4 % ( 11 nt) | 53.1 % ( 14 nt) | 100.0 % (301 nt) |
| *RNAi-SlGADall* | 76.2 % ( 17 nt) | 100.0 % (143 nt) | 86.9 % ( 26 nt) |

**Fig. S1**
Design of the RNAi-targeted regions. (A) Schematic illustrations of *SlGAD* cDNAs. Black bars indicate the position of the RNAi-targeted regions used for *RNAi-SlGAD1*, *RNAi-SlGAD2*, *RNAi-SlGAD3* and *RNAi-SlGADall* constructs. Numbers above the cDNAs indicate the nucleotide sequence position. (B) Nucleotide identities between the RNAi-targeted regions and the corresponding region of *SlGADs.* The maximum length of the consecutive nucleotides that matched completely between the RNAi-targeted regions and the corresponding region of *SlGADs* are also shown. UTR, untranslated region; CDS, coding sequence; nt, nucleotides.

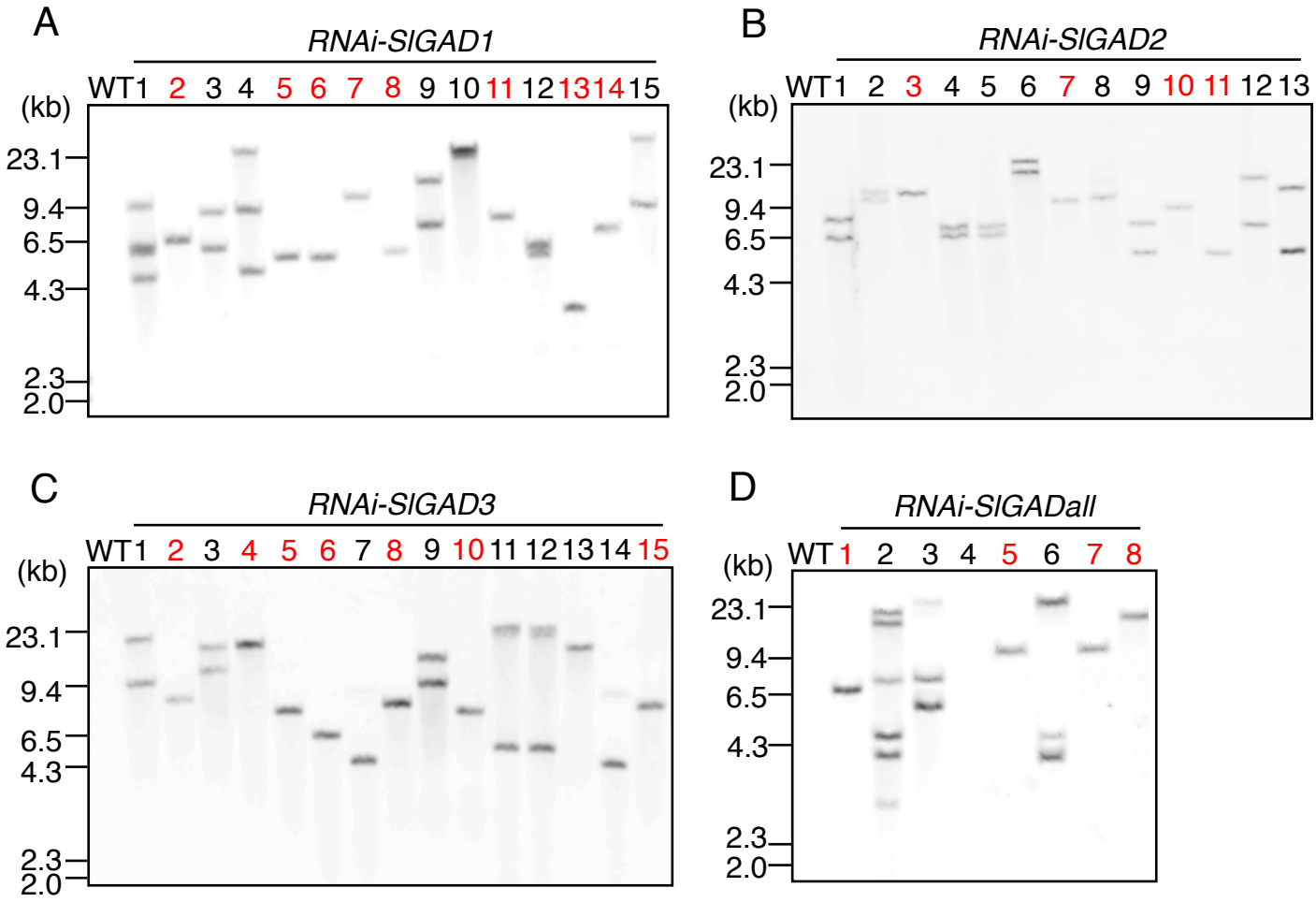



**Fig. S2**
Southern blot analysis in $T_0$ RNAi lines. Genomic DNA (10 µg) was digested with *Eco*RI and detected with the *NPTII* probe. Lane numbers show independent transgenic lines of each construct; *RNAi-SlGAD1* (A), *RNAi-SlGAD2* (B), *RNAi-SlGAD3* (C) and *RNAi-SlGADall* (D) . WT was also tested as a negative control. The lines contained single-copies of T-DNA insertion are shown in red.

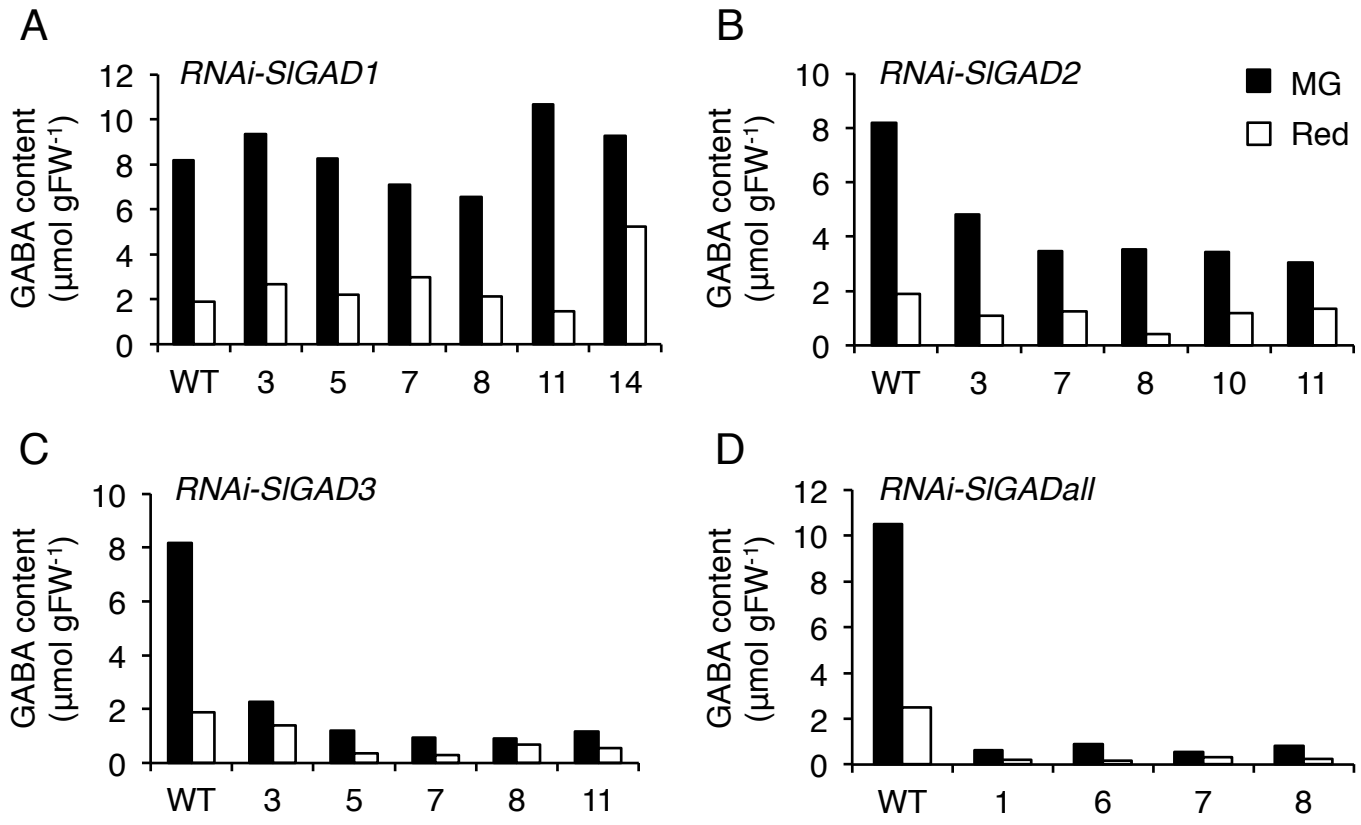



**Fig. S3**

GABA content in fruits in $T_0$ RNAi lines. The GABA content in MG fruits and red fruits of *RNAi-SlGAD1* (A), *RNAi-SlGAD2* (B), *RNAi-SlGAD3* (C) and *RNAi-SlGADall* (D) were determined. The numbers below the horizontal axis indicate the independent transgenic lines of each construct. WT fruits were also analyzed as a control.

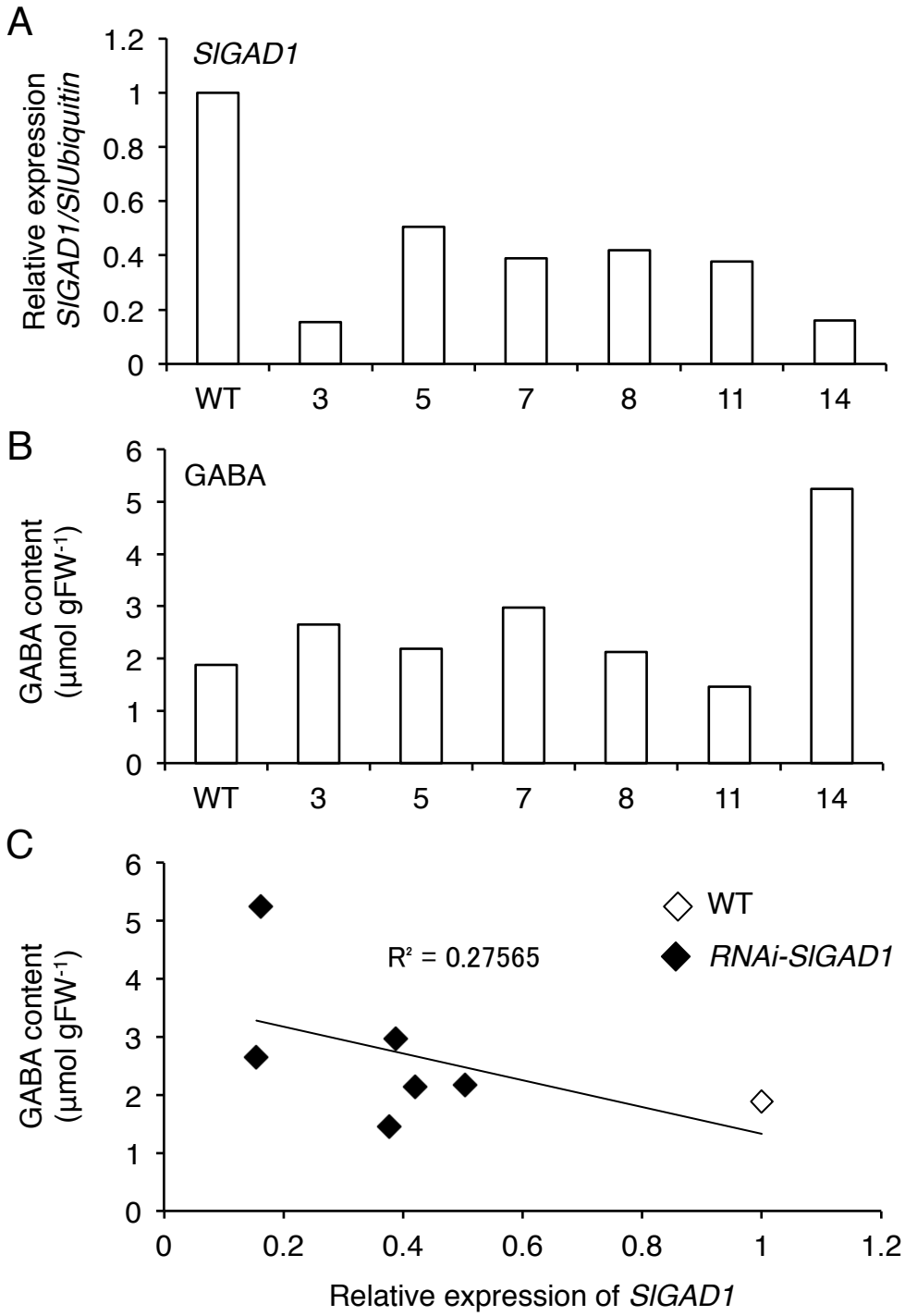



**Fig. S4**
Effects of *SlGAD1* suppression on the fruit GABA levels in $T_0$ *RNAi-SlGAD1* lines. Relative expression levels of *SlGAD1* (A) and GABA contents (B) in the red fruits of $T_0$ *RNAi-SlGAD1* lines and WT. (C) Correlation between *SlGAD1* expression levels and GABA contents in the red fruits of $T_0$ *RNAi-SlGAD1* lines and WT.

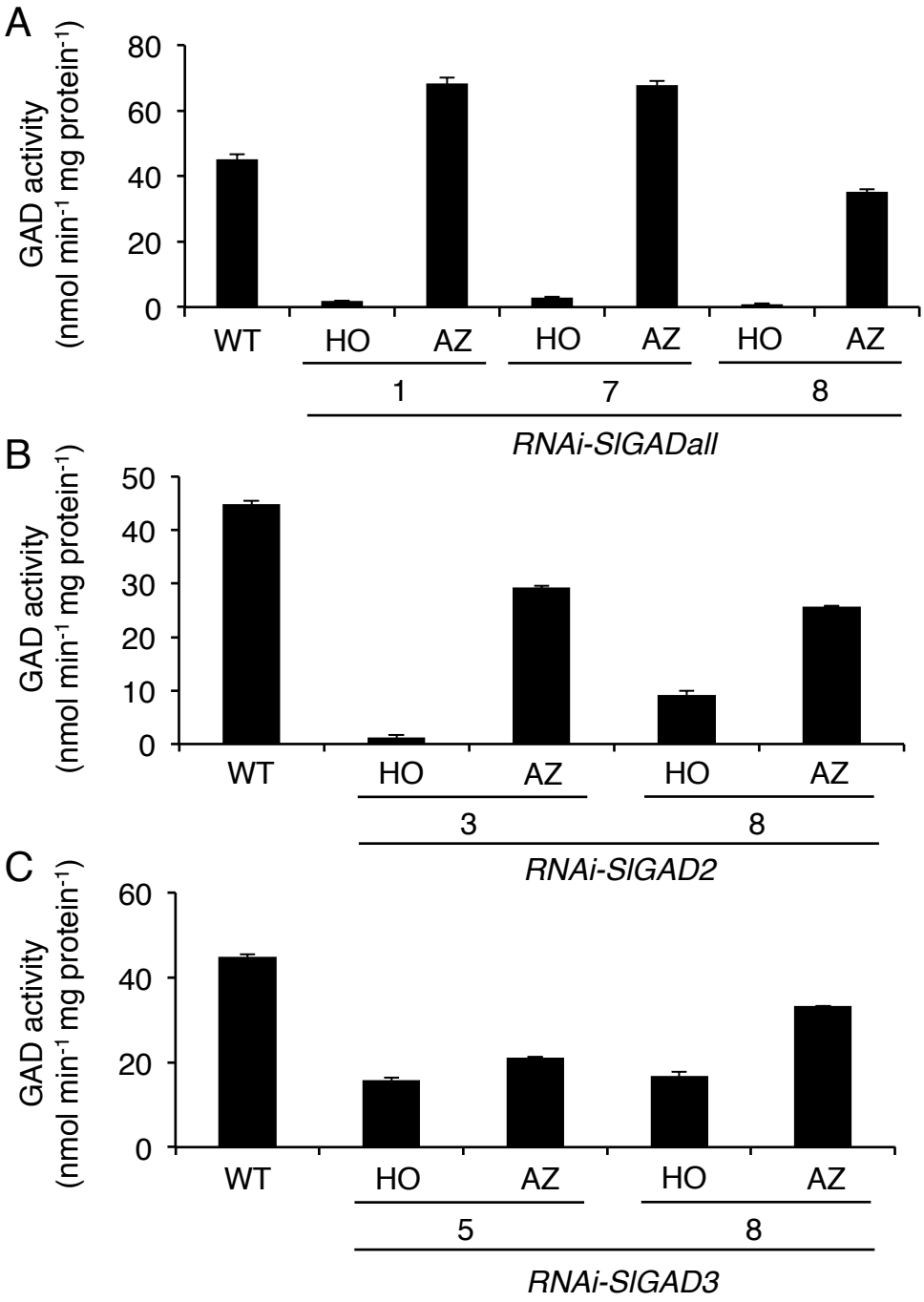



**Fig. S5**
GAD activities in the MG fruits of transgenic lines. (A) $T_3$ *RNAi-SlGADall*. (B) $T_2$ *RNAi-SlGAD2*. (C) $T_2$ *RNAi-SlGAD3*. The mean values of three technical replicates are shown. Separate experiments were done at least three times for each construct.

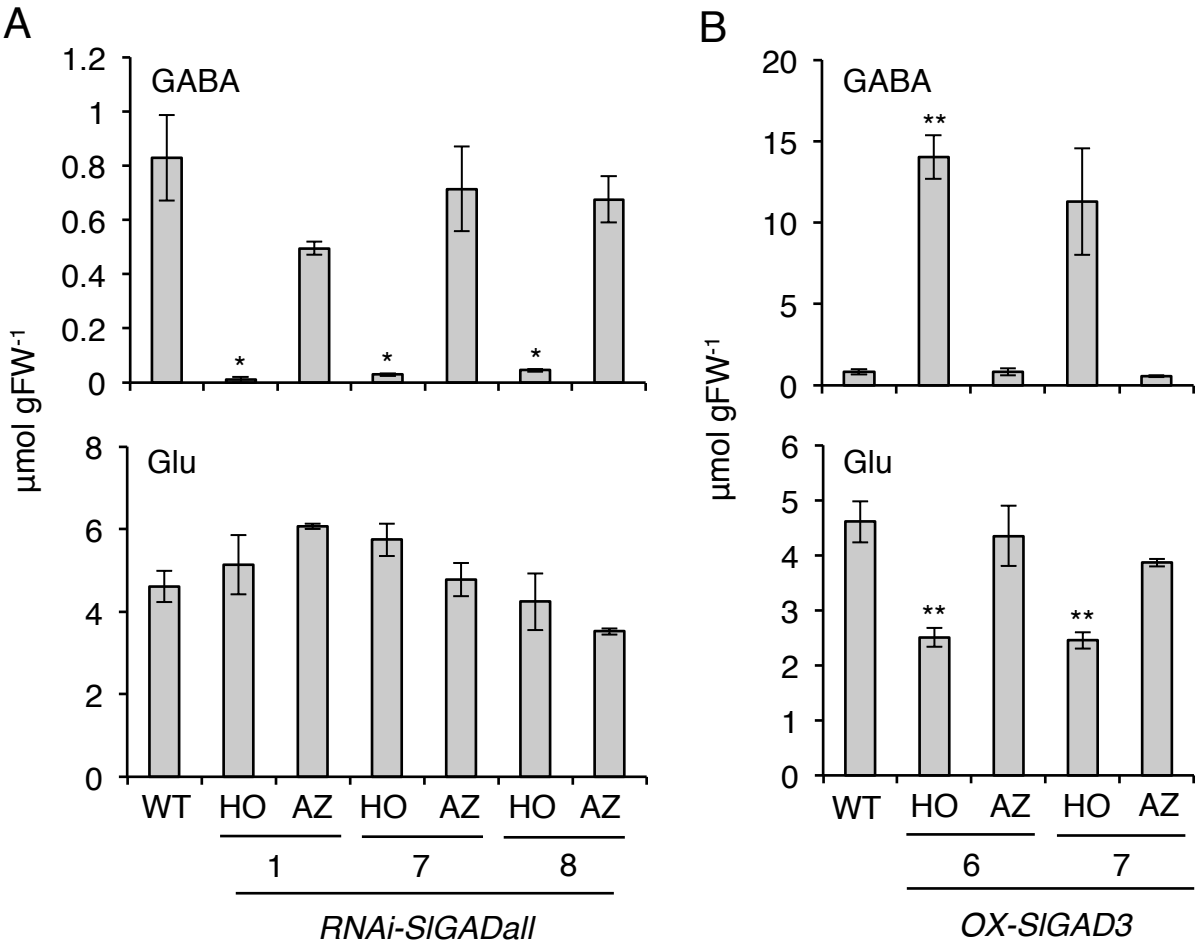



**Fig. S6**

GABA and glutamate levels in the transgenic tomato leaves. GABA (upper column) and glutamate (lower column) levels in the leaves from 22-day-old $T_5$ *RNAi-SlGADall* (A) and $T_3$ *OX-SlGAD3* (B) are shown. All results are the mean±SE (n=3). Asterisks indicate a significant difference between WT and HO, or WT and AZ according to Student's *t*-test (**P*<0.05, ***P*<0.01). WT, wild-type; HO, homozygous; AZ, azygous; FW, fresh weight.

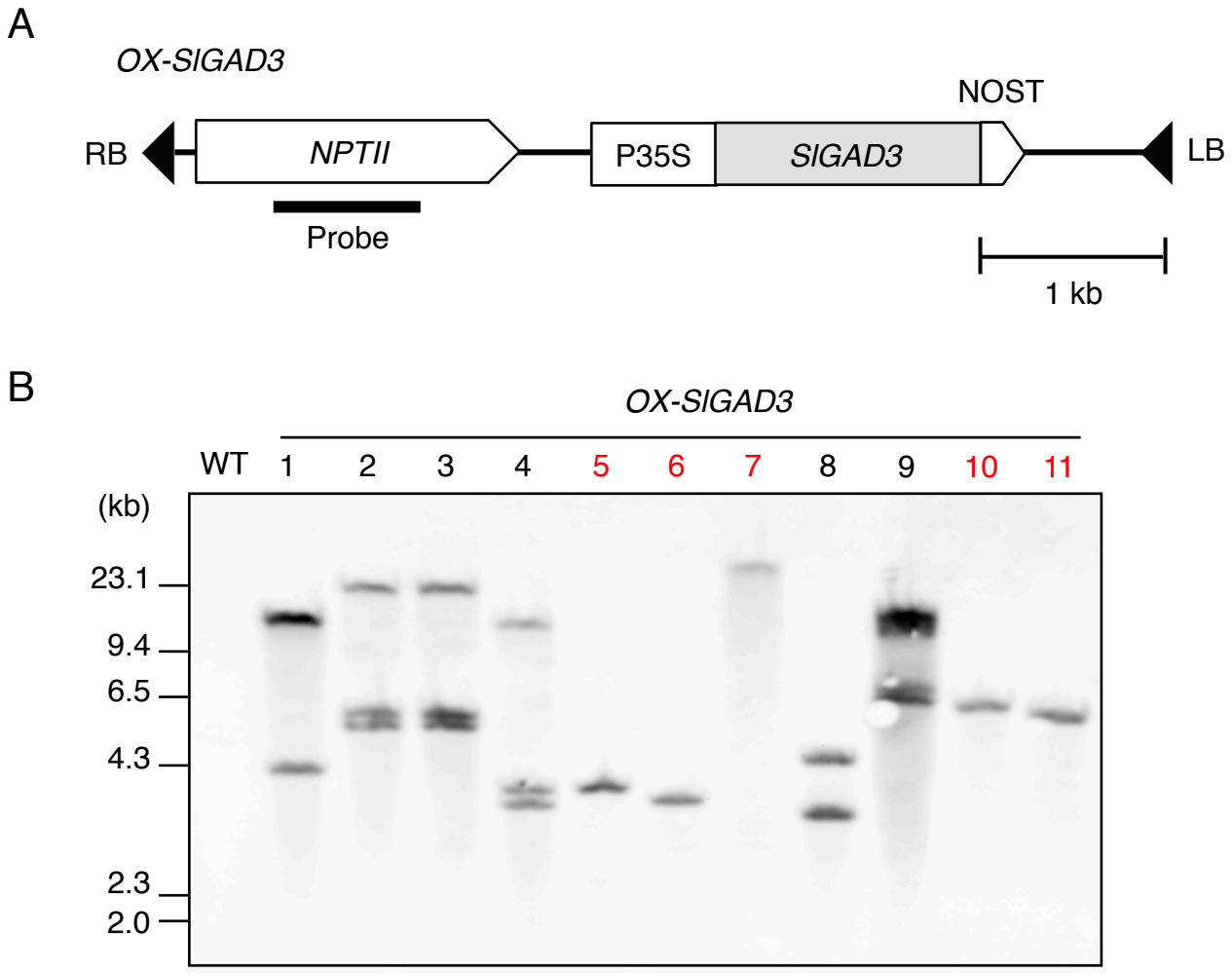



**Fig. S7**
Generation of *SlGAD3* over-expressed lines driven by CaMV 35S. (A) T-DNA maps used for tomato transformation. RB and LB, right and left borders of T-DNA; *NPTII*, neomycin phosphotransferase II gene; P35S, cauliflower mosaic virus 35S promoter; NOST, nopaline synthase gene terminator. (B) Southern blot analysis in the $T_0$ generation of regenerated plants. Genomic DNA (10 μg) was digested with *Eco*RI and detected with the *NPTII* probe. Lane numbers show independent transgenic lines of *OX-SlGAD3*. WT was also tested as a negative control. The lines contained single-copies of T-DNA insertion are shown in red.

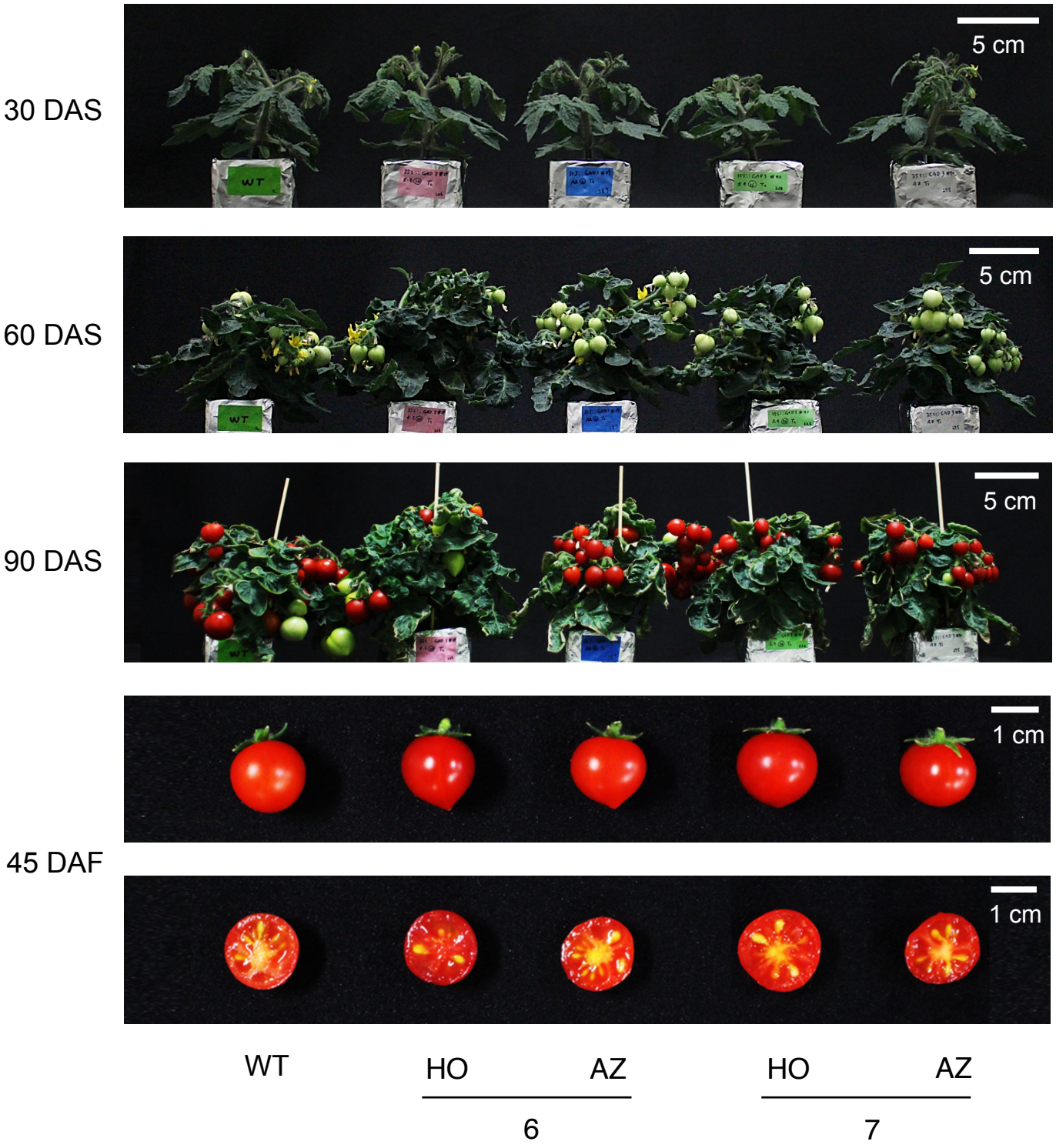



**Fig. S8**
Plant and fruit phenotypes of $T_2$ *OX-SlGAD3.* Two independent homozygous lines of *OX-SlGAD3* (6- and 7-HO) were grown with the corresponding azygous lines (6- and 7-AZ) and WT. The phenotypes of whole plants at 30, 60 and 90 DAS and fruits at 45 DAF are shown.

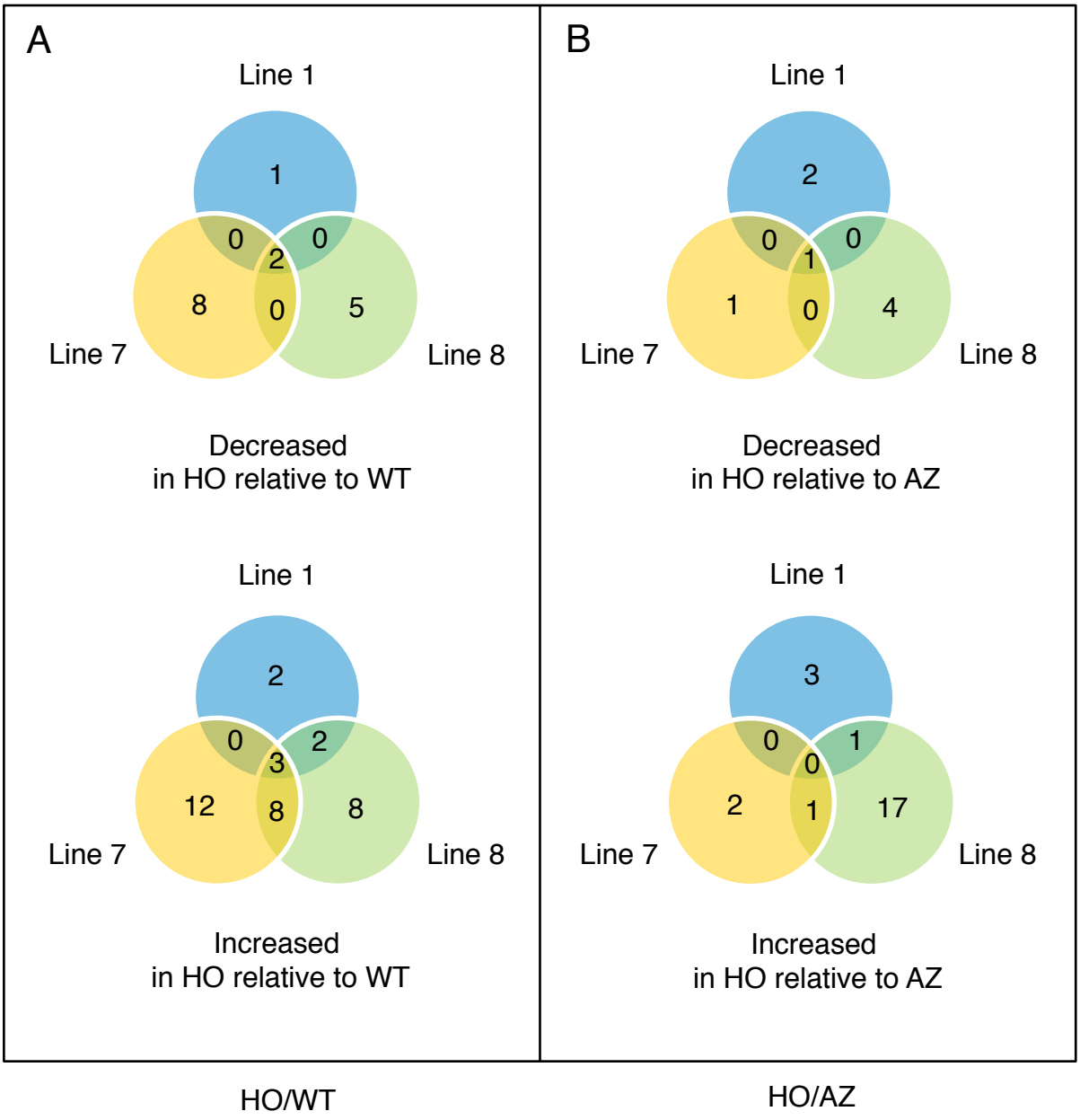



**Fig. S9**
Metabolite changes in MG fruits of *RNAi-SlGADall* lines. The metabolite contents in MG fruits of $T_3$ *RNAi-SlGADall* homozygous lines (1-, 7- and 8-HO) were compared with that of WT or the corresponding azygous lines (1-, 7- and 8-AZ). Venn diagrams indicate the number of metabolites which significantly down- or up-regulated in homozygous lines relative to WT (HO/WT; A) or homozygous lines relative to azygous lines (HO/AZ; B). In the calculation of HO/AZ ratio, each metabolite level in lines 1-, 7- and 8-HO were divided by that in lines 1-, 7- and 8-AZ, respectively.

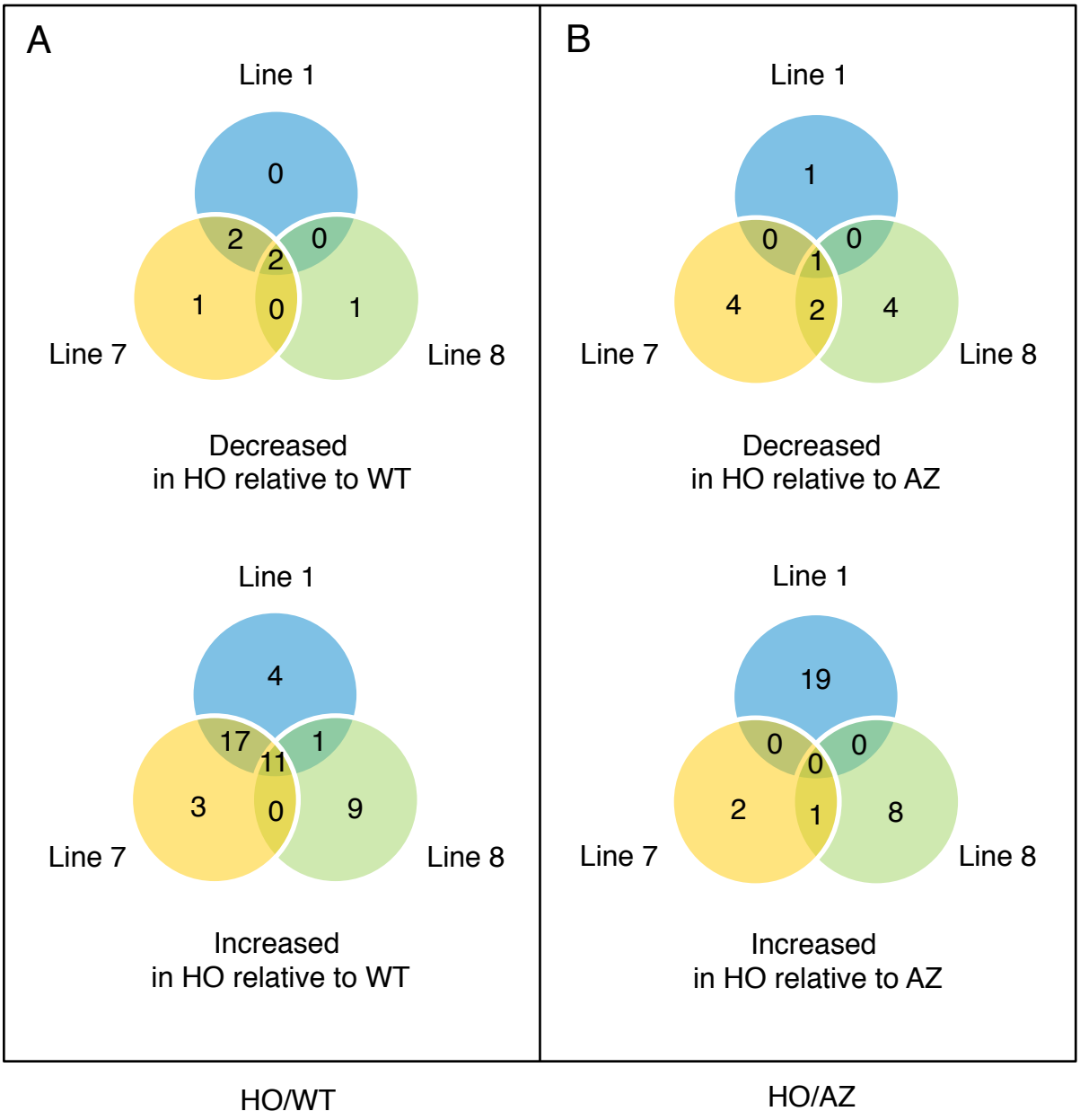



**Fig. S10**
Metabolite changes in red fruits of *RNAi-SlGADall* lines. The metabolite contents in red fruits of $T_3$ *RNAi-SlGADall* homozygous lines (1-, 7- and 8-HO) were compared with that of WT or the corresponding azygous lines (1-, 7- and 8-AZ). Venn diagrams indicate the number of metabolites which significantly down- or up-regulated in homozygous lines relative to WT (HO/WT; A) or homozygous lines relative to azygous lines (HO/AZ; B). In the calculation of HO/AZ ratio, each metabolite level in lines 1-, 7- and 8-HO were divided by that in lines 1-, 7- and 8-AZ, respectively.

**Table S1** List of primers used in this study

| Used for | Primer name | Primer sequence (5'→3') |
|---|---|---|
| Vector construction of *RNAi-SlGAD1* | *SlGAD1*-1,526F | GAGGAAGTGCGTGATGACAA |
| | *SlGAD1*-1,849R | TTATGCCCGGATTGATTCAT |
| Vector construction of *RNAi-SlGAD2* | *SlGAD2*-1,529F | GCATGGAAGAAGTTTGTTGCT |
| | *SlGAD2*-1,840R | TGAAACAAGGTGACATGACATTA |
| Vector construction of *RNAi-SlGAD3* | *SlGAD3*-1,305F | CATGTTACAGTGTTGCGCGTT |
| | *SlGAD3*-1,605R | CACATTTTTTATAATTAATAC |
| Vector construction of *RNAi-SlGADall* | *SlGAD2*-283F | CAGAATGTGACAAATTGAT |
| | *SlGAD2*-424R | AGTTTCTCCATCTTCAAGTGG |
| Vector construction of *OX-SlGAD3* | *SlGAD3*-75F | ATGGTTCTCTCAAAAACTCCTTCTG |
| | *SlGAD3*-1,529R | CTAACAAATAGATGCTTTCCTAGCT |
| Creation of probe for southern blot analysis | *NPTII*-F | ATGATTGAACAAGATGGATTGCAC |
| | *NPTII*-R | TCAGAAGAACTCGTCAAGAAGGCG |
| qRT-PCR of the internal control | *SlUbiquitin*-F | CACCAAGCCAAAGAAGATCA |
| | *SlUbiquitin*-R | TCAGCATTAGGGCACTCCTT |
| qRT-PCR of *SlGAD1* | *SlGAD1*-12F | AAACTTCCCATTTCCCAACC |
| | *SlGAD1*-117R | CGATTGATCGGAGGAGAAAA |
| qRT-PCR of *SlGAD2* | *SlGAD2*-35F | CTTTGATCTTCTCCGTCGTTG |
| | *SlGAD2*-139R | ATATCGAGACGCGAAAGTCG |
| qRT-PCR of *SlGAD3* | *SlGAD3*-1,150F | CAGGACGTTTCAATATAATC |
| | *SlGAD3*-1,259R | CCTACGGAGGGTCTCAGAG |

**Table S2**

Characteristics of plant growth and fruit development in $T_3$ *RNAi-SlGADall* (1-, 7- and 8-HO), the corresponding azygous lines (1-, 7- and 8-AZ) and WT.

| | WT | 1-HO | 1-AZ | 7-HO | 7-AZ | 8-HO | 8-AZ |
|---|---|---|---|---|---|---|---|
| Days to flowering [a] | 30.3±0.2 | 29.5±0.2 * | 31.0±0.3 | 30.2±0.3 | 30.0±0.4 | 30.7±0.3 | 30.8±0.4 |
| Plant height (cm) [a] | 5.5±0.2 | 5.7±0.2 | 5.4±0.2 | 4.4±0.1** | 4.0±0.2** | 6.9±0.2** | 6.9±0.2 ** |
| Number of leaves (/plant) [a] | 6.7±0.2 | 5.7±0.2 ** | 5.8±0.3 * | 6.3±0.2 | 6.3±0.2 | 6.2±0.3 | 6.3±0.3 |
| Fruit weight (g FW) [b] | 2.6±0.2 | 2.8±0.2 | 2.2±0.1 | 4.1±0.2 ** | 2.7±0.2 | 3.1±0.2 * | 3.0±0.1 |

The plant height and the number of leaves were measured when the first flower opened.
The fruit weight was measured at 45 DAF
[a] Values are the mean±SE (n=6)
[b] Values are the mean±SE (n=25)
Asterisks indicate a significant difference between WT and HO, or WT and AZ according to Student's *t*-test (*$P$<0.05, **$P$<0.01)

**Table S3**

Characteristics of plant growth and fruit development in $T_2$ *OX-SlGAD3* (6- and 7-HO), the corresponding azygous lines (6- and 7-AZ) and WT.

| | WT | 6-HO | 6-AZ | 7-HO | 7-AZ |
|---|---|---|---|---|---|
| Days to flowering [a] | 31.0±0.0 | 32.3±0.4 * | 32.3±0.6 * | 32.8±0.2 ** | 30.3±0.4 |
| Plant height (cm) [a] | 4.5±0.2 | 5.5±0.4 * | 4.8±0.2 | 4.2±0.1 | 4.3±0.2 |
| Number of leaves (/plant) [a] | 5.4±0.2 | 6.3±0.2 ** | 5.8±0.3 | 5.8±0.2 | 5.7±0.3 |
| Fruit weight (g FW) [b] | 2.6±0.2 | 2.5±0.1 | 3.9±0.2 ** | 2.6±0.2 | 2.4±0.1 |

The plant height and the number of leaves were measured when the first flower opened.
The fruit weight was measured at 45 DAF
[a] Values are the mean±SE (n=6)
[b] Values are the mean±SE (n=25)
Asterisks indicate a significant difference between WT and HO, or WT and AZ according to Student's *t*-test (*$P$<0.05, **$P$<0.01)