The use of *SlAdh2* promoter as a novel fruit-specific promoter in transgenic tomato

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Abstract Fruit-specific promoters play an important role in the improvement of traits, such as fruit quality through genetic engineering. In tomato, the development of fruit-specific promoters was previously reported, but less attention has been paid to the promoters involved in the fruit development stage. In this study, we characterized the gene expression patterns of tomato alcohol dehydrogenase 2 (*SlAdh2*) in various tissues of wild-type tomato (cv. Ailsa Craig). Our findings revealed that *SlAdh2* expression levels were higher in the developing fruit than in the leaves, stems, and flowers. The *ProSlAdh2* region, which is expressed at different stages of fruit development, was isolated from tomato genomic DNA. Following this, it was fused with a β -glucuronidase reporter gene (*GUS*) and introduced into wild-type tomato using *Agrobacterium*-mediated transformation to evaluate promoter activity in the various tissues of trans-

genic tomato. The *ProSlAdh2:GUS* promoter exhibited strong activity in the fruit and weak activity in the stems, but displayed undetectable activity in the leaves and flowers. Interestingly, the promoter was active from the appearance of the green fruit (1 cm in size) to the well-ripened stage in transgenic tomatoes, indicating its suitability for transgene expression during fruit development and ripening. Thus, our findings suggest that *ProSlAdh2* may serve as a potential fruit-specific promoter for genetic-based improvement of tomato fruit quality.

Keywords Fruit quality, Genetic transformation, Gene expression, GUS activity, Promoter analysis, Tomato

Introduction

Tomato (*Solanum lycopersicum*) is one of the most commonly eaten fruits in the world due to its attractive color, flavor, and versatility; it is also a rich source of vitamins, minerals, and antioxidants that are associated with resistance to disease (Clinton et al. 1996; Levy et al. 1995). To meet consumer demand, many researchers have attempted to improve the quality of tomato fruit using genetic engineering (Butelli et al. 2008; Dharmapuri et al. 2002; Le et al. 2006; Mollet et al. 2008; Wang et al. 2008). In particular, the cauliflower mosaic virus 35S promoter (*CaMV 35S*) has been widely used as a constitutive promoter for the expression of foreign genes in tomato (de Jong et al. 1994; Dutt et al. 2014; Hiwasa-Tanase et al. 2012; Speirs et al. 1998). However, the expression of the *CaMV 35S* promoter is influenced by the physiological and developmental stages of tomato, and it not always appropriately expressed in transgenic tomato depending on the species (de Jong et al. 1994; Dutt et al. 2014). Thus, it is important to select specific promoters according to the specific tissue in which the

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foreign gene is to be expressed. In tomato, the ethylene response genes *E8* and *E4* have been used as fruit-specific promoters (Coupe and Deikman 1997; Deikman et al. 1998; Xu et al. 1996), but their expression has been observed only in the late fruit ripening stage. Indeed, fruit-specific promoters that are expressed throughout the entire fruit development and ripening process have yet to be reported.

Alcohol dehydrogenases (Adh) play an important role in the production of aroma volatiles in tomato fruit (Speirs et al. 1998; Speirs et al. 2002). Of the genes encoding Adh enzymes, *SlAdh2* is specifically expressed in fruit tissue in a developmentally regulated manner (Longhurst et al. 1994; Manríquez et al. 2006; Speirs et al. 2002), and its over-expression has been reported to enhance the flavor of transgenic tomato fruit by increasing the alcohol and Z-3-hexenol levels (Speirs et al. 1998). Therefore, the possible use of *SlAdh2* promoter as a fruit-specific promoter in studying of fruit quality using genetic engineering is worthy of research attention. In this respect, the β -glucuronidase gene (*GUS*) reporter system is a reliable method for the functional analysis of promoters with respect to gene expression in transgenic plants (Atkinson et al. 1998; Wang et al. 2005).

In this study, we cloned the promoter region of the *SlAdh2* gene and conducted promoter analysis using the *GUS* expression system. This study revealed that *SlAdh2* is regulated at the transcriptional level throughout the entire fruit development process (i.e., during the immature, mature, and late stages), and thus *SlAdh2* promoter has the potential to be useful as a novel fruit-specific promoter for the improvement of traits related to fruit quality in tomato via genetic transformation.

Materials and Methods

Plant material

Wild Tomato cv. Ailsa Craig (*Solanum lycopersicum*) were grown in greenhouses equipped with heating and cooling systems, with supplementary lighting for a total of 16 h days (27°C) and 8 h nights (19°C) and supplied with slow release fertilizer. The leaves, stems, flowers, and fruits were collected at different developmental stages as samples for RNA isolation and gel-blot analysis. The fruit were collected at nine stages of maturity: 1-cm-sized fruit, early-immature green (EIM; 10 days after the appearance of the 1-cm fruit), late-immature green (LIM; 20 days after the appearance of the 1-cm fruit), mature green (MG; 25 days after the appearance of the 1-cm fruit), breaker (B; 28 days after the

appearance of the 1-cm fruit), and 3 days, 7 days, 10 days, and 15 days after the appearance of the breaker stage. In addition, the early and fully flowering stages were collected. Three biological samples were collected for each stage. The samples were collected and frozen immediately in liquid nitrogen, followed by storage at -80°C until further analysis.

RNA isolation and gel-blot analysis

To analyze the expression patterns of *SlAdh2*, total RNA was isolated from the three biological samples of the various tissues using TRI Reagent™ solution (Ambion, USA) (Naing et al. 2017), and the RNA was quantified using a spectrophotometer. Approximately 20–30 µg of total RNA was resolved on 1% (w/v) agarose gel with 7.5% (v/v) formaldehyde and blotted onto a Hybond N membrane (Amersham Biosciences, Piscataway, NJ, USA). The membranes were then transferred and baked at 80°C for 2 h, followed by pre-hybridization for 3 h using the membrane supplier's protocol and hybridization at 65°C with ³²P-labeled random primed DNA probes for 16 h. The filters were washed in 2 X SSC, 0.1% SDS and then 1 X SSC, 0.1% SDS at 65°C. Signal intensity was visualized using autoradiography with Kodak X-OMAT-AR film and two intensifying screens at -80°C (Chung et al. 2010).

ProSlAdh2 sequence identification

SlAdh2 (CP023762.1) was identified from the Sol Genomics database and cross-checked with the NCBI database. The promoter region was retrieved 5' upstream of the identified *SlAdh2*, and the promoter sequence was characterized using PlantCARE software.

Construction and transformation of the *ProSlAdh2::GUS* fusion

To construct the promoter/*GUS* reporter system, the *ProSlAdh2* was obtained from the wild tomato cv. Ailsa Craig genomic DNA with PCR using the forward primer; *ProSlAdh2* tttgcgac ATTACAAATATGTACTATAAT (underlined sequence: appended *sall* site for cloning) and the reverse primer; *ProSlAdh2* ttccggg CTCTTGATTTTTTTT TAGTGAT (underlined sequence: appended *smal* site for cloning). The PCR conditions were as follows: pre-denaturation for 5 min at 94°C, amplification for 30 cycles at 94°C for 1 min, annealing for 1 min at 58°C and extension for 2 min at 72°C, with a final extension at 72°C for 10 min using Pfu Ultra DNA polymerase (Stratagene, now Agilent, <http://>

Fig. 1 Construction of the *ProSlAdh2::GUS* fusion using pBI101 vector. RB, right border; LB, left border; Nos-pro, nopaline synthase promoter; NPT-II, neomycin phosphotransferase; Nos-ter, nopaline synthase terminator

www.genomics.agilent.com).

The PCR DNA product was combined with pBI101-GUS plasmid using the *Sal*I and *Sma*I restriction enzymes (Fig. 1). Recombinant plasmids were introduced into *Agrobacterium tumefaciens* LBA4404, and transformation was conducted using the wild-type (WT) tomato cv. Ailsa Craig according to the Boyce Thompson Institution transformation protocol (Van Eck et al. 2006). Successful transformants were selected by rooting them in Murashige and Skoog basal medium (MS) containing 100 mg/L of kanamycin sulfate, followed by PCR detection using specific primers. Approximately 25 transformants that were PCR-positive were observed and the number of copies inserted into the T_0 lines was further confirmed using Southern blot analysis, following the method described by Yang et al. (2005). Of these, some T_0 lines exhibiting a single copy were selected for the generation of homozygous T_2 lines. The selected T_0 lines were self-pollinated to generate T_1 lines, and the continuous self-pollination of T_1 lines was employed to generate homozygous T_2 lines; the T_1 and T_2 lines were selected by culturing them in MS basal media containing kanamycin sulfate and using specific primers (data not shown). Finally, the T_2 lines were further confirmed using southern blot analysis and the lines (*ProSlAdh2* #2, #21, and #24) were used for GUS analysis.

Histochemical detection of GUS

To measure GUS activity using histochemical analysis, the tomato tissues were stained with a buffer containing 0.5 mM X-Gluc (Melford Laboratories, Chelsworth, UK) and

incubated in the dark at 37°C for 1 ~ 16 h. The tissues were infiltrated with fixatives and staining solutions under reduced pressure for 2 ~ 5 min. Longitudinal sections and cross-sections of the tissues were fixed in 0.3% formaldehyde in 50 mM sodium phosphate (pH 7.0) and 1 mM EDTA for 30 min and washed 4 ~ 5 times with 50 mM sodium phosphate (pH 7.0) prior to staining in a buffer consisting of 100 mM sodium phosphate (pH 7.0), 1 mM EDTA, 0.05% Triton X-100, 0.1 mM $K_3[Fe(CN)_6]$, and 0.1 mM $K_4[Fe(CN)_6]$. Prior to imaging, thin sections were submerged O/N in a 2:1:1 mixture of chloral hydrate, lactic acid, and phenol (CLP). Images were taken under tungsten light with Fuji RTP727 film using a Leica WildM10 stereomicroscope (Blume and Grierson 1997).

Results and Discussion

Expression of *SLAdh2* in wild-type tomato cv. Ailsa Craig

The importance of Adh enzymes in the formation of flavor volatiles has been identified in tomato (Longhurst et al. 1994; Tanksley 1979), and the gene encoding this enzyme (*SLAdh2*) has been reported to be regulated during the fruit development and ripening stages (Speirs et al. 1998). In the present study, we investigated the expression patterns of *SLAdh2* in various organs (leave, stems, flowers, and fruit at various stages of maturity) in WT tomato cv. Ailsa Craig using Northern blot analysis. The results shown in Figure 2 reveal that *SLAdh2* was not expressed in the leaves and in the two stages of flowering (early and fully flowering), while

Fig. 2 Analysis of *SLAdh2* gene-expression patterns in wild-type tomato in relation to the developmental stage. L, leaf; S, stem; EF, early flower; FF, full-blooming flower; 1cm, 1-cm fruit; EIM, 10 days after the appearance of 1-cm fruit; LIM, 23 days after the appearance of the 1-cm fruit; MG, mature green; B, breaker; B3, 3 days after the breaker stage; B7, 7 days after the breaker stage; B10, 10 days after the breaker stage ; B15, 15 days after the breaker stage. 18S RNA bands were used as standards.

it was detected in the stems.

Its expression was higher in the fruit during the development stages (1-cm green to MG) than in the stems, with maximum expression observed at the EIM stage. In the later stages (B to B15), the expression of the gene was transcriptionally higher than in the initial stages, with the highest expression levels reached 7 and 10 days after the breaker stage (Fig. 2). This high expression of *SlAdh2* during the fruit development and ripening stages in tomato is supported by the findings of Speirs et al. (1998). The higher expression of the gene during fruit ripening, especially after the breaker stage, is likely due to the higher

Adh activity, which is positively regulated during fruit ripening (Speirs et al. 2002).

ProSlAdh2 sequence identification and characterization

SlAdh2 have been characterized for tissue-specific expression during different developmental stages, as indicated above. To confirm its potential use as a promoter, we first identified and verified *SlAdh2* using the Sol Genomics and NCBI databases (<https://solgenomics.net/>) and then retrieved the promoter region 5' upstream (2078-bp sequence length; Fig. 3). The *SlAdh2* gene and promoter sequence were sub-

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-2078 ATTACAAATATGTACACTATAATATTATCTCAATATCCAAGACTAGTACAGAAAAAATTG
      CAAT-box      TATA-box
-2018 ATTTGAATTGGACACCAATTTTGAAGTAGTATACTAACTGTTGCTAGTAATTAACAT
      CAAT-box      CAAT-box      TATA-box
-1958 GCACCAATTGAAGCTTCTAGGCAGCAACTGTCAGCACATAACATGTAAGTAATTGGTTT
      MBS
-1898 AAGTGGGTTTATGTTTATGTCTAACTTTATTTTTTAAAGGAAAAAGTCAAACTATTTC
-1838 TTGAATTAGGAAAAAGTCCAAAAAATATCTCTATTATGTTTGTCTAAAAATATC
-1778 TTCTTTTTTAATTTATATATATCTTTTCATTACCTTTTGTACTTATATCCTTTC
      TATA-box      TATA-box
-1718 ATTCACCTTTTTTTTACTTAACCTATACCTTCCCTCATATTATTAAATAAAAAATTA
      chs-CMA1s      TATA-box      TATA-box
-1658 TATATATATATATATCTTCAAGACTCTAATTTTTTAAAAAAGAAATCGAATAATTTTCA
      TATA-box      Box 4
-1598 TATTTTCCTTAATTTATTTTAAAAAGAAATTTTTTAGATAATTAATATATAATA
      TATA-box
-1538 ATAAATATTATTAGTGCCCAATATCTATCTCTTTCTAAATAAGTAAAAAGAAAAA
-1478 AATGTTAACTTAATTATTTTTTCCCTAATATTGATACTACTTTTTAAAGAAAAAGTTTC
-1418 ACACCAATTTTACTTAAAGATTAACTATTAAAAAATGAAAGAGAAAAATACCAGAT
      chs-CMA1s
-1358 GAATAGTATTTGTAATAAAATAATATATTGTTATGAGTATGACATAAATTTTTTTT
      TATA-box
-1298 TAAAAATATTAATCATCTATATAATGTTTAGAATCTTGATTAAATTAATTAATATTAGCC
      TATA-box      Box 4      TATA-box      Box 4
-1238 GTTAATTTTTCTTTTGTATGAGTTTTCAATCAAGTAAAACTTAAGAAAAATGGAGAG
-1178 GGAGTTGGATCCAAAAATAAATAAATAAAAAATTACAATATTTTTTTTATATGAAGA
      TATA-box
-1118 AATCGGGTCTTGAAGAAGTAAATGTTTGTATTGTTTAAATAATGATAAATAATCTAA
      TATA-box      GTI-motif
-1058 TTAAGGTGGTCTTTTACTACACATAAATAATTTTTAAAAATAAAAAATAAGAGAA
      TATA-box
-998 AATTGTTAATTTCAAGAAATATAGTGAGCGAAAAAGATGAATAAAGAAATTTTTAGATC
      TATA-box
-938 AAAAGATGAAGTAAAGATAATATTTTTGATATTTTACCTTTTTTTTATATACGTTGT
      CAAT-box      ARE      TATA-box
-878 TAAGATACTCAAGGACCAATTTATAAGAAACCAACCAAGGTTTCATGAAAGATTGAA
      TATA-box
-818 AAAACCATCAACCAACGCAATCAACTGCTCAACTAGAATCAACATACAAAAAATATA
      MBS
-758 CTTAATGAGATCATTATAACCTAAATTATAACCCCTCCGTCACCTTTCATTATCATGT
      MRE      TATA-box
-698 TATATTTTACGCAAGTCAATTTGATTCATTTTAAAAAGTTAAATGAGATTATATTAATTA
      TATA-box
-638 ATATTTTAAATAAAATTTTCAGATATTTAAAAATATATGAAAAGTATCATGAATTGTAG
      TATA-box      TATA-box
-578 TTTTTTTTGCATATATGAAAAATACATTATAAATATTAGTCAATTTTTTTTATAAATTG
      TATA-box
-518 ACTCTAAATATGAAAAAATGACAATTAAAAATAGACGGAGTTGTAAATTAGCTTATT
-458 AATTATTAAATTGATAAATATCATAATTAACTGATAATGACAATTAAATATTAGAAGAC
-398 GATAATGACAGAAATCAACGTTATTTTAGGTATAATTTTTGTTGTATTTTTGAAAAAAT
      AT-rich element
-338 AATCTTTTTCTGCAACTGTTATATTAAGTGAACAAACAAAAACAGTAGTATAAAAA
      MBS
-278 AATTACAAGTGGACATAAAACAAATGAGATACAGTATTGTGTTTCCATTGGAATATTA
-218 GCTTGACAAAACTCAACGAGCAACACAAAAACACAGCTAAAAAACCTGTTTGAATA
-158 ATCCAGTGACCAAAACATGTAATGGTTTTACTGTGGCTTATTGTTTTTACACCTTCCC
      TATA-box
-98 AATTATAAATATCCACTGCCTCAACTGAGTAACAAACCAAAATTTGTGTTCTATAAAAG
      CAAT-box      MBS      TATA-box
-38 TTTTCATATTAGTGATCACTAAAAAATCAAGAAGATG

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Fig. 3 The DNA sequence and *cis*-acting element of the fruit-specific *ProSlAdh2* region. Nucleotides are numbered on the left side, with ATG at position +1

sequently characterized using PlantCARE software.

Construction and transformation of the *ProSLAdh2::GUS* and the histochemical detection of GUS

The *ProSLAdh2::GUS* fusion was identified based on the 5' upstream region of *SLAdh2* with the restriction enzymes *Sall* and *smaI* at 5' and 3' respectively in the genomic DNA. The *ProSLAdh2::GUS* fusion was transferred into WT tomato leaf segments using *Agrobacterium*-mediated gene transformation. The transformants expressing the promoter were identified using a gene-specific primer (data not shown). For promoter analysis, T₀ lines with a single copy of *ProSLAdh2* were selected to generate homozygous T₂ lines, and some of these T₂ lines were further confirmed using southern blot analysis (Fig. 4) and the T₂ lines (*ProSLAdh2#2*, *ProSLAdh2#21*, and *ProSLAdh2#24*) and WT specimens were then selected.

Stems, leaves (young and old), flowers (sepals and petals), and fruits (maturity stages 1 to 9) were collected from WT

Fig. 4 The number of copies of *ProSLAdh2::GUS* in T₂ transgenic tomato plants confirmed using Southern blot analysis

and from the selected lines (*SLAdh2#2*, *SLAdh2#21* and *SLAdh2#24*). The activity of *ProSLAdh2::GUS* in the collected samples was analyzed using histochemical assays. The assays revealed no promoter activity in any of the leaf or flower stages in any of the T₂ lines (*SLAdh2#2*, *SLAdh2#21*, and *SLAdh2#24*) or the WT (Fig. 5), whereas activity was observed in both stem sections (longitudinal and cross-sections) of the T₂ lines but not the WT (Fig. 6).

Line #21 exhibited stronger activity in both stem sections

Fig. 5 GUS activity assays for the leaves and flowers of transgenic tomato expressing *ProSLAdh2::GUS*

Fig. 6 GUS activity assays for the stem sections of transgenic tomato expressing *ProSLAdh2::GUS*

Fig. 7 GUS activity assays for the fruit development stage of transgenic tomato expressing *ProSlAdh2::GUS*

Fig. 8 GUS activity assays for the fruit ripening stage of transgenic tomato expressing *ProSlAdh2::GUS*

than the other selected lines. Promoter activity was also observed in the fruits of the T₂ lines, though this activity varied with the developmental stages. Low *ProSlAdh2::GUS* activity was detected in the 1-cm fruits of the T₂ lines, with a gradual increase starting from 10 days after the appearance of the 1-cm fruits up to the breaker stage in all three lines (Fig. 7).

After the breaker stage, strong promoter activity was still observed in the three lines, particularly from 7 days to 15 days after the breaker stage (Fig. 8). Our assay results suggest the upregulation of *SlAdh2* in the later stages of fruit development in all lines. These results support the vital role of Adh in the production of flavor volatiles in fruit because a positive correlation between Adh levels and the

flavor of tomato fruit has been reported (Speirs et al. 1998; Speirs et al. 2002). Taken together, our results indicate that *SlAdh2* is useful as a fruit-specific promoter for the improvement in the quality of tomato fruit and that the overexpression of *SlAdh2* using genetic engineering could help to enhance the flavor of tomato fruit.

Conclusion

We analyzed the expression patterns of *SlAdh2* in various WT tomato tissues and found higher expression levels during the fruit development and ripening stages than in the other tissues (i.e., the leaves, stems, and flowers). Similarly, in-

creases in the activity of the *ProSlAdh2::GUS* were detected during fruit development, particularly in the later stages. In addition, because the selected *SlAdh2* promoter region contains various important conserved promoter sequences, it can be used as a dynamic tool for the analysis of developmental stages and fruit ripening. Moreover, metabolic genetic engineering of *SlAdh2* gene could help to enhance the flavor of tomato fruit.

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