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細胞壁結合型酸性インベルターゼ遺伝子 Wiv-1 の果実特異的発現によるトマト果実糖蓄積への影響

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Effect of Fruit-specific Expression of the Cell-Wall-Bound Acid Invertase Gene *Wiv-1* on Hexose Accumulation in Tomato Fruits

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I Introduction

Soluble sugar content is an important breeding objective because it contributes to the eating and processing quality of various fruit crops, including tomato. In these crops, various sugars and organic acids are stored in the vacuoles. The cultivated species of tomato (*Solanum lycopersicum*) accumulates mainly hexoses, whereas some wild relatives of tomato (*S. chmielewskii*, *S. peruvianum*, *S. habrochaites*, and others) accumulate mainly sucrose (Miron and Schaffer, 1991; Stommel, 1992; Yelle et al., 1991). Sucrose is the main sugar translocated in most plants, including tomato (Yamaki, 2010). The sucrose synthesized in source leaves is loaded into the phloem by the H^+–sucrose co-transporter (Riesmeier et al., 1994), is translocated into the fruit through the phloem, and is then unloaded into sink cells via a symplastic pathway (plasmodesmata) or an energy-dependent apoplastic pathway (Roitsch and Gonzalez, 2004; Yamaki, 2010). Sucrose unloaded into the apoplast from the sieve elements can be cleaved by cell-wall-bound (extracellular or apoplastic) acid invertase (CWI; Roitsch and Gonzalez, 2004; Yamaki, 2010). Hexoses (glucose and fructose) generated by CWI are then transported into the sink cells by one or more hexose transporters (Roitsch and Gonzalez, 2004; Ruan and Patrick, 1995; Ruan et al., 1997).

In tomato, four paralogous genes for CWI (*Lin5*, *Lin6* (*Wiv-1*), *Lin7*, and *Lin8*) have been identified (Godt and Roitsch, 1997), and have been characterized extensively (Jin et al., 2009; Zanor et al., 2009). One gene has been isolated independently by two research groups using different methods, but has been confirmed to be the same gene: *Wiv-1*, which was isolated by Ohyama et al. (1998), is the same gene as *Lin6*, which was previously isolated by Godt and Roitsch (1997). Among the four CWI genes, expression of *Lin5* has been reported to be fruit-specific (Godt and Roitsch, 1997) and *Wiv-1* expression is observed in vascular tissues (Ohyama et al., 2006), but its expression is very low in the fruits (Godt and Roitsch, 1997; Ohyama et al., 1998). Fridman et al. (2000) reported that a quantitative trait locus (QTL) for high soluble sugar content in tomato fruits contains a fruit-type CWI gene (*Lin5*); the high-sugar allele was derived from a wild relative of tomato (*S. pennellii*). Fridman et al. (2004) suggested that the high-sugar trait from the wild species is due to a difference in enzyme kinetics, probably derived from amino acid sequence changes caused by single-nucleotide polymorphisms (SNPs) detected at the *Lin5* locus. RNAi-mediated knockdown of hexose transporter genes (McCurdy et al., 2010) or *Lin5* (Zanor et al., 2009) caused a reduction of hexose accumulation in tomato fruits. Further, elevation of CWI activity by RNAi inhibition of an invertase inhibitor gene caused increases in seed weight and hexose levels in the fruit (Jin et al.,

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2009). These important results suggest that sugar accumulation in tomato fruit occurs in part through an apoplastic pathway. However, an important point must still be clarified: can a specific increase in the level of a sucrose-cleaving enzyme in the apoplast of sink organs such as fruits enhance sugar transport and elevate hexose levels? To answer the question, we combined the cDNA for *Wiv-1* (Ohyama et al. 1998) with a young-fruit-specific promoter for a tomato sucrose synthase (SS) gene, *TOMSSF* 5' (Ohyama et al., 2010), and introduced the construct into a dwarf tomato cultivar ('Micro-Tom'). SS gene promoters isolated from tomato and Japanese pear (*Pyrus serotina*) both showed expression specific to young tomato fruits in promoter–*GUS* experiments (Ohyama et al., 2010). The level of expression of the promoter *TOMSSF* 5' from tomato seemed to be higher than that of the promoter *PypSUS1* 5' from Japanese pear; this might be at least partially due to the presence of an intron located downstream of the start of transcription site of *TOMSSF* (Ohyama et al., 2010; see also Fig. 1). By comparing the sugar contents and gene expression in the transformants, we assessed the effects of the construct on sugar accumulation and the physiological roles of CWI in sugar metabolism.

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Ⅱ Materials and Methods

1 Construction of *TOMSSF* 5′ ::*Wiv-1*

The binary vector was constructed by replacing the β-glucuronidase (*GUS*) gene and the *35S* promoter of pBI121 (Jefferson et al., 1987) with the *Wiv-1* cDNA (Ohyama et al., 1998) and *TOMSSF* 5' (the tomato fruit SS promoter; Ohyama et al., 2010), respectively (Fig. 1). The vector was introduced into *Rhizobium radiobacter* (formerly *Agrobacterium tumefaciens*) by electroporation (Sambrook and Russell, 2001).

2 *Rhizobium*-mediated transformation of tomato and selection of true transformants

Tomato (*S. lycopersicum* 'Micro-Tom'; Meissner et al., 1997) was transformed as described previously (Ohyama et al., 1995) with *R. radiobacter* LBA4404 harboring the construct *TOMSSF* 5' ::*Wiv-1* (Fig. 1). Regenerated plants were grown in a greenhouse at 15 to 25 °C under natural day length during the autumn and winter in Mie,

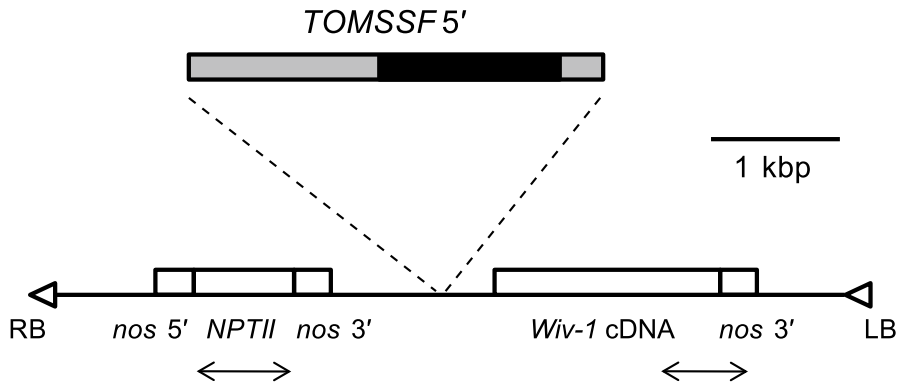

Fig. 1 Structure of the binary vector.
The black box indicates an intron approximately 1.6 kbp in length near the 3' end of the *TOMSSF* 5' sequence (Ohyama et al., 2010). PCR screening to identify transformants was done at the positions indicated by double arrows (see Materials and Methods). LB and RB indicate the left and right borders of the T-DNA, respectively. *nos* 5', *NPTII*, and *nos* 3' are the promoter of the nopaline synthase gene, the neomycin phosphotransferase coding region, and the nopaline synthase 3' untranslated region, respectively (Jefferson et al., 1987).

Japan. Transformation with the complete T-DNA was confirmed by means of PCR using two primer pairs (see also Fig. 1): Wiv-1 FS1 (5'-GGTGGTCTTGGGCCTTTTGGG-3') and Tnos (5'-ATCATCGCAAGACCGGCAAC-3') to detect the *Wiv-1* cassette, and NPT-1 (5'-CAAGATGGATTGCACGCAGG-3') and NPT-2 (5'-GAAGAACTCGTCAAGAAGGCG-3') to detect the *NPTII* cassette. The PCR reaction followed an initial 30 s at 94 ˚C; 35 cycles of 30 s at 94 ˚C, 1 min at 55 ˚C, and 1 min at 72 ˚C; and a final 5 min at 72 ˚C. PCR products were analyzed by electrophoresis in 1.2% agarose gel.

3 Northern blot analysis of *Wiv-1* expression

Transformed 'Micro-Tom' fruits were sampled at an early stage (20 to 30 days after anthesis [DAA]) and then stored at –80 ˚C. Extraction of total RNA from the fruit, RNA electrophoresis, northern blotting, and hybridization were performed as described previously (Ohyama et al., 2010). PCR labeling of the cloned *Wiv-1* cDNA (Ohyama et al., 1998) with DIG (Roche Diagnostics GmbH, Mannheim, Germany) was done using the M13 universal primers M4 (5'-GTTTTCCCAGTCACGAC-3') and RV (5'-CAGGAAACAGCTATGAC-3') according to the manufacturer's protocol.

4 Real-time PCR analysis

Total RNA was extracted from individual fruit halves (the other half of each fruit was used for sugar measurements, as described below) using an RNeasy Plant Mini Kit with an RNase-Free DNase Set (Qiagen K.K., Tokyo, Japan). First-strand cDNA was synthesized using the ThermoScript RT-PCR System (Life Technologies Japan Ltd., Tokyo, Japan) with an oligo $(dT)_{20}$ primer and the total RNA samples used as templates. Quantitative real-time PCR analysis was performed with a QuantiTect SYBR Green PCR Kit (Qiagen) and a LightCycler (Roche). The reaction mixture (20μL), containing specific primers and appropriate template DNA (1:10-diluted first-strand cDNA), was prepared according to the manufacturer's protocol. A primer pair (W1488F, 5'-CCGATGCCTCAAGGTCAAG-3', and W1631R, 5'-CAGCACCAAAACTTTCCACTATC-3') was used for amplification of the cDNAs (144 bp in length) derived from both the endogenous and introduced *Wiv-1* mRNAs (DDBJ/GenBank/EMBL accession numbers AF506004 and AB004558). This primer pair was designed from sequences flanking the 4th and 5th introns of *Wiv-1* to avoid amplification of contaminating genomic fragments of the *Wiv-1* gene (data not shown). For amplification of mRNAs (cDNAs) of tomato actin (DDBJ/GenBank/EMBL accession number AW217630) and *Lin5* (Godt and Roitsch, 1997; DDBJ/GenBank/EMBL accession numbers AY173050 and AJ272304, respectively), we used the primers T-actin-F1 (5'-CCCAAAGGCTAATCGTGAAA-3') and T-actin-R1 (5'-GACCACTGGCATACAGTGAGAG-3' ; product length of 107 bp), and the primers Lin5F1537 (5'-GAAGCAATGTACAAGCCCTCA-3') and Lin5R1668 (5'-TATGCATGTTTTGCCACCAG-3' ; product length of 132 bp), respectively. Lin5F1537 and Lin5R1668 were designed from regions flanking the 5th intron of *Lin5* to avoid amplification of contaminating genomic fragments of *Lin5* (data not shown).

After the mixture was preheated at 95 ˚C for 15 min, a thermal cycle consisting of 15 s at 94 ˚C, 20 s at 55 ˚C, and 8 s at 72 ˚C was repeated 60 times, with no final extension. The amount of cDNA was determined using a set of diluted plasmid DNAs (1 to 0.0001 pg) as external standards: pλZWI65-2 for *Wiv-1*; p5M21 for *Lin5*, amplified using Lin5F1537 and Lin5R1786 (5'-TACATGCATCCATGCTCCAA-3'; DDBJ/GenBank/EMBL accession number AB695289); and pm1 for tomato actin, amplified using LeACTIN-F (5'-ATTCCCTGACTGTTTGCTAGT-3') and LeACTIN-R (5'-TCCAACACAATACCGGTGGT-3' ; DDBJ/GenBank/EMBL accession number AB695290). The levels of *Wiv-1* and *Lin5* expression (measured by the amount of cDNA produced) were normalized to that of the tomato actin gene.

5 Measurement of the soluble sugar content

Soluble sugars were extracted from 100- to 200-mg aliquots from a single fruit of each line by grinding the fruit with a mortar and a pestle in 1 mL of distilled water, and proteins in the extracts were removed by the addition of 9 μL of 20% (w/v) 5-sulfosalicylic acid solution, followed by centrifugation (20,000 × *g*, 10 min). The sugar content in each filtered extract was measured by high-performance liquid chromatography, as described previously (Ohyama et al., 1995).

Ⅲ Results

1 Introduction of a chimeric gene into tomato

The *Wiv-1* binary vector (Fig. 1) was used for transformation of tomato ('Micro-Tom') by *Rhizobium*. Regenerated plants were screened by PCR using two primer pairs to identify transformants. The transgene was found in 15 lines (verified by PCR), which were used for subsequent analyses (Table 1, Figs. 2–4). Putative nontransformants found to lack *Wiv-1* by PCR (this study) were used as controls (3 lines in total; Figs. 2, 3). All transformants showed normal growth, flower setting, fruit color, ripening period, and single-fruit fresh weight (data not shown).

2 Northern blotting analysis of the young fruits of transformants

As the *TOMSSF* 5' promoter used in the *TOMSSF* 5' ::*Wiv-1* construct is specific to young fruits (Ohyama et al., 2010; Wang et al., 1994), the *Wiv-1* expression levels in young fruits of the transformants were analyzed (Fig. 2). The levels of *Wiv-1* mRNA in the transformed lines were higher than those in the control lines, indicating that the *TOMSSF* 5' ::*Wiv-1* construct achieved overexpression of *Wiv-1* mRNA.

3 Quantification of invertase gene expression in young fruits

Figure 3 shows the expression levels of *Wiv-1* mRNA in young fruits (20 to 30 DAA) of each transformed line. Nine (1-4, 1-13, 1-J, 2-2, 2-8, 2-11, 2-17, 3-9, and 3-19) of the 15 transformed lines showed higher expression levels than the average of the three control lines (C1-3). The levels for 1-48 and the control C1 line disagreed with the northern results (Fig. 2), probably because of differences in the maturity between the fruits used for the northern analysis and the real-time PCR analysis.

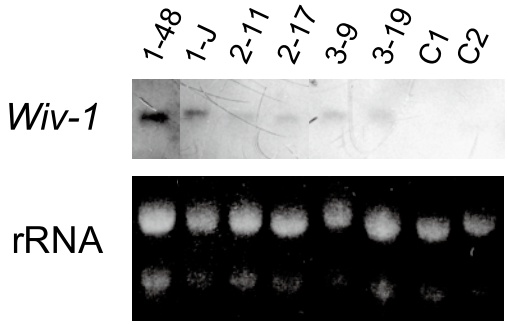

Fig. 2 Northern blot analysis of young 'Micro-Tom' fruits transformed with the *TOMSSF* 5' ::*Wiv-1* construct.

Total RNAs were extracted from fruits at 20 to 30 DAA, electrophoresed under denaturing conditions, and blotted onto a nylon membrane, and then the *Wiv-1* mRNA was detected as described in the Materials and Methods. The rRNA bands on the same gel were visualized by ethidium bromide staining as a loading control. Each lane contains total RNA from the fruit of an independent transformant (1-48, 1-J, 2-11, 2-17, 3-9, and 3-19) or a control nontransformant (C1 and C2).

4 Correlation analysis

Correlations between the levels of *Wiv-1* mRNA and the sugar contents in young fruits of the transformants were significant (Table 1, Fig. 4). The fruits used for measurement of the sugars were the same as those used for mRNA quantification (Fig. 3). Differences in soluble sugar contents between the control and transformed plants were not dramatic

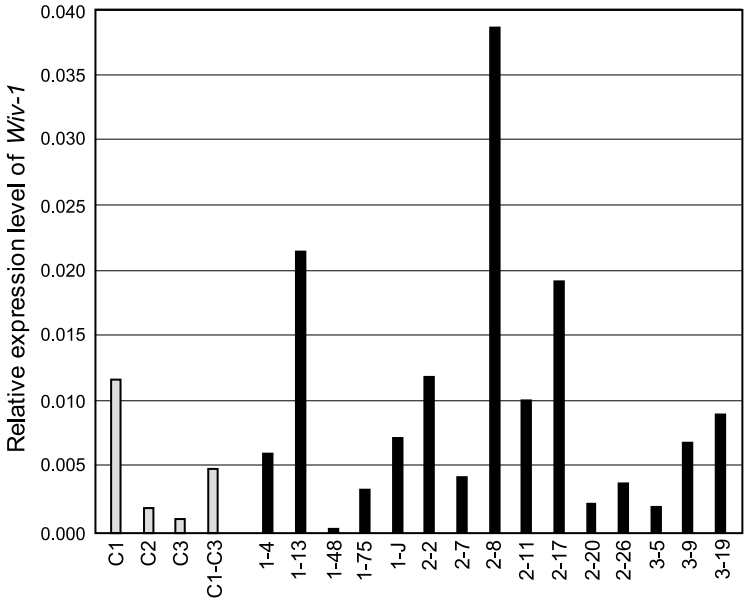

Fig. 3 Relative expression levels of *Wiv-1* (based on mRNA levels) in young fruits of lines transformed with the *TOMSSF* 5' ::*Wiv-1* construct.
C1, C2 and C3 are the levels in control nontransformants, and C1–C3 means the average value of the levels in the three nontransformants.

(data not shown). The correlations tended to be higher for lines with lower expression levels (Table 1, Fig. 4).

As the expression of *Lin5* is fruit-specific (Godt and Roitsch, 1997), we analyzed whether compensatory changes in the *Lin5* mRNA level occurred in the transformants. We found no significant correlations between the levels of *Lin5* mRNA and *Wiv-1* mRNA in young fruits of the transformants (r = 0.134), or between the levels of *Lin5* mRNA and the sugar contents (r = 0.176 for sucrose, r = 0.135 for hexoses, and r = 0.146 for total sugars).

Table 1 Correlations between *Wiv-1* expression (based on mRNA levels) and the contents of soluble sugars in young fruits of the transformants

	Correlation coefficient	
	Relative expression level[a] of *Wiv-1*[b] is 0.0002 to 0.0120 (n = 12)[c]	Relative expression level[a] of *Wiv-1*[b] is 0.0002 to 0.0400 (n = 15, total)[c]
Sucrose	0.462	0.232
Hexoses	0.679*	0.527*
Total sugars	0.662*	0.495

[a] Normalized to the level of tomato actin mRNA.
[b] The primers that we used did not distinguish between the endogenous gene-derived mRNA and the transgene-derived mRNA.
[c] Number of lines.
* Significant at $P < 0.05$.
The raw data are also displayed in a scatterplot (Fig. 4).

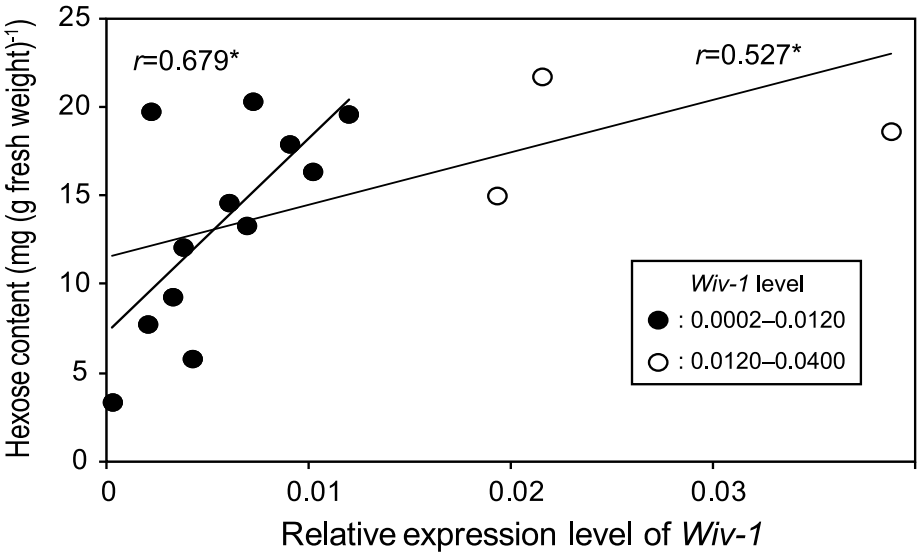

Fig. 4 Scatterplot of the relative expression level of *Wiv-1* (based on mRNA levels; see also Table 1) and hexose content in young fruits transformed with the *TOMSSF* 5' ::*Wiv-1* construct.
r: correlation coefficient; *Significant at $P < 0.05$.

Ⅳ Discussion

We introduced a chimeric *Wiv-1* construct, *TOMSSF* 5' ::*Wiv-1*, into 'Micro-Tom' (Fig. 1). No detrimental effects on plant growth, such as those found in tobacco transformants that expressed a yeast invertase gene driven by the constitutive 35S promoter (Sonnewald et al., 1991), were observed in the transformants. The level of *Wiv-1* mRNA was positively correlated with hexose content in young fruits of the transformants (Table 1, Fig. 4).

Why did we observe positive correlations among the hexose contents and the *Wiv-1* mRNA levels in young fruits of the transformants? It is known that the sugar unloading process involves two pathways: sucrose from sieve elements enters the sink organs with extracellular hydrolysis (apoplastic pathway via hexose transporters) or without extracellular hydrolysis (mainly the passive symplastic pathway via plasmodesmata) by CWI (Roitsch and Gonzalez, 2004; Ruan and Patrick, 1995). Several authors have suggested that an apoplastic pathway using CWI is involved in the unloading process in tomato fruits (Jin et al., 2009; McCurdy et al., 2010; Ruan and Patrick, 1995; Zanor et al., 2009). The absence of correlation of *Wiv-1* and *Lin5* mRNAs suggests that no compensatory changes in the level of *Lin5* mRNA had occurred in the transformants. It is likely that most of the fluctuation in the level of *Wiv-1* mRNA in fruits of the transformants (Fig. 3) was caused by overexpression of the mRNA driven by the *TOMSSF* 5' promoter (Fig. 2). The promoter of the tomato SS gene *TOMSSF* is strongly expressed in the vascular tissues of young fruits (Ohyama et al., 2010). Considering the present results together with previously published data, we suggest that the positive correlations between the hexose and the *Wiv-1* mRNA levels observed in young fruits of the *TOMSSF* 5' ::*Wiv-1* transformants (Table 1, Fig. 4) were probably achieved by promotion of the apoplastic hexose transport during the early stages of fruit development, through an increase in sucrose degradation activity in the apoplasts of vascular tissues (probably phloem). The correlations between the mRNA level and the hexose contents in young fruits of transformants were higher at lower levels of *Wiv-1* mRNA (Table 1, Fig. 4), which suggests that extreme expression of *Wiv-1* is not necessary to increase fruit hexose content. The fact that CWI is an enzyme with a low Michaelis constant, K_m (Karuppiah et al., 1989), may support this hypothesis. Furthermore, the lack of correlations between the levels of *Wiv-1* mRNA

and the sucrose content (Table 1) may indicate that most of the sucrose transported into the fruit's cytosol is derived from the symplastic pathway without extracellular hydrolysis.

Even though the apoplastic pathway is active in young fruit, it's unclear why there was no correlation among the hexose contents and the natural fluctuation of *Lin5* mRNA levels in young fruits of the transformants. One possibility is that the *TOMSSF* promoter (used for the construct) drives more favorable gene expression than does the native promoter of *Lin5*.

One limitation of our study is that the results were obtained only from the T_0 generation of transformants. To further characterize the sugar accumulation mechanisms in tomato fruit, it will be necessary to re-evaluate the transformed lines for both their mRNA levels and their enzyme levels after the transgenes are fixed (made homozygous). It will also be necessary to introduce the *TOMSSF* 5' ::*Wiv-1* construct into normal tomato cultivars, rather than dwarf cultivars such as 'Micro-Tom'. Furthermore, in addition to the young-fruit-specific promoter, it will be necessary to analyze the effects of a ripening-related phloem promoter construct to analyze the effects of CWI on sugar content in ripening fruits.

Summary

Because soluble sugars are an important component of tomato fruit quality, we investigated the possible role of a key enzyme, cell-wall-bound acid invertase (CWI), in sugar accumulation in tomato fruit. We fused cDNA for *Wiv-1*, a gene encoding tomato CWI, to a young-fruit-specific tomato sucrose synthase (SS) gene promoter, and introduced the chimeric construct into tomato ('Micro-Tom'). Significant positive correlations were found between the level of *Wiv-1* expression and the hexose content in young developing fruits of the transformants. These results suggest that the hexose transport in young fruits was promoted by the expression of *Wiv-1*, probably through an increase in the level of sucrose degradation in the apoplast.

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細胞壁結合型酸性インベルターゼ遺伝子 *Wiv-1* の果実特異的発現によるトマト果実糖蓄積への影響

大山 暁男・布目 司・福岡 浩之

摘 要

糖含量はトマト果実の品質を左右する重要な要素である．そこで我々は，トマト果実の糖蓄積における鍵酵素の1つ，細胞壁結合型酸性インベルターゼ（CWI）の生理的役割について調査した．細胞壁結合型酸性インベルターゼ cDNA *Wiv-1* を，若い果実に特異的な発現を示すトマトショ糖合成酵素遺伝子プロモーターと連結し，得られたキメラ遺伝子をトマト（マイクロトム）に導入した．その結果，形質転換体の若い果実においては，*Wiv-1* 発現レベルと還元糖量との間に有意な正の相関が見出された．以上の結果は，形質転換体の若い果実において，*Wiv-1* 遺伝子の発現に起因するアポプラスト部のショ糖分解レベルの増大を介して果実中への還元糖輸送が促進されたことを示唆している．

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