Organ- and stage-specific expression of the lectin gene in tomato

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ABSTRACT

To elucidate the physiological roles of lectin in tomato (*Solanum lycopersicum*), the structure and expression of the corresponding lectin gene were investigated in a Japanese large tomato cultivar Momotaro-Yoku. The gene seemed intronless and the deduced amino acid sequence of the encoded protein was identical to that of the reported cherry tomato lectin CTL. The lectin gene was expressed more in the fruits at the mature green/orange stage than in the immature green and red stages. In a model tomato variety Micro-TOM, the tomato lectin mRNA and protein were detected only in fruit but not in leaf, stem, or root. The amount of transcript during ripening was highest at the orange stage in Micro-TOM as well. Ripening-specific gene expression provides some insight into the roles of lectin in tomato.

INTRODUCTION

Tomato (*Solanum lycopersicum*) lectins are chitin-binding proteins that have been purified from the fruits.[6,11-13,20] They are used as classic tools in the study of basic and applied biology such as analyses of glycosylation of lysosomal membrane glycoproteins in sublines of human colon cancer[22] and the development of drug delivery systems.[1] In spite of their deep contribution to glycobiology, little is known about the physiological roles of lectins in tomato plants, which is cultivated in many parts of the world. In contrast to chitin-binding lectins in other plants, tomato lectin does not seem to be involved in defense against fungi and insects, because they exhibit none of the antifungal and insecticidal activities that are often observed in other chitin-binding lectins in plants.[2,7]

Oguri *et al.*[16] revealed the molecular structure and properties of the tomato lectin by cloning its corresponding cDNA from unripe cherry-tomato fruits. The encoded protein contains four hevein domains whose arrangement is distinctive from that in potato lectin.[25] As the first step for elucidating the biological roles of lectin in tomato, we have, in parallel with their study, investigated the structure of the genomic gene for a tomato lectin in a Japanese large tomato cultivar Momotaro-Yoku. Based on the gene structure, we were able to investigate the expression modes of the gene in Momotaro-Yoku and additionally in a model tomato variety Micro-TOM. The results clearly demonstrate the organ- and stage-specific expression of the tomato lectin gene.

MATERIALS AND METHODS

Plant materials and growth conditions. Fruits of a tomato (*Solanum lycopersicum*) cultivar "Momotaro-Yoku", which were used throughout this study, were kindly provided by Otsuka Chemical Co., Ltd, Tokushima, Japan. Seeds of a model tomato variety "Micro-TOM" were transferred from Dr. Kou Aoki at Kazusa DNA Research Institute, Chiba, Japan. Micro-TOM was cultivated and fruits were harvested as described by Iijima *et al.*[3] Stems, leaves, and roots of plants 40-days after flowering were used, when almost all fruits had entered into the stage of red overripe.[9]

Purification of tomato lectin. Tomato fruits (Momotaro-Yoku) were processed in a blender in the presence of phosphate buffered saline (PBS),[23] and centrifuged (8,000 rpm, 25 min, 4ºC). Proteins in the supernatant were precipitated in 55% saturated $(NH_4)_2SO_4$, harvested by centrifugation (8,000 rpm, 25 min, 4ºC), dissolved in 10 mM phosphate buffer (pH 5.9), and dialyzed against the same buffer. The desalted protein solution was subjected to a column (10 mm x 80 mm) filled with a cation-exchange resin CM-Toyopearl (Tosoh, Tokyo, Japan). Proteins were eluted in a linear NaCl gradient (0-100 mM) and subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE; see below for details). Fractions containing

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a protein of *ca*. 100 kDa, which corresponded to previously reported tomato lectin,[12,13] were collected and dialyzed against 10 mM phosphate buffer (pH 5.9), and subjected to a high-resolution cation-exchange column Resource S (GE Healthcare UK Ltd., Buckinghamshire, UK) chromatography. Proteins were eluted in a linear NaCl gradient (0-100 mM). The fractions were subjected to SDS-PAGE to check for protein homogeneity.

Protein analysis. Protein concentration was measured by Lowry's method,[10] using bovine serum albumin (BSA) as a standard protein. Proteins were separated on a 7.5% poly- acrylamide gel containing 0.1% SDS (SDS- PAG)[8] and were visualized by staining SDS- PAGs with Coomassie Brilliant Blue (CBB) R-250. For immuno-blot analysis, tomato organs were processed in a blender in PBS and centrifuged (8,000 rpm, 25 min, 4ºC). Proteins included in the supernatants were separated by SDS-PAGE and blotted onto a polyvinylidene difluoride membrane (Immobilon-P; Millipore). Tomato lectin was detected using *anti*-CTL (cherry tomato lectin) antiserum.[13] To determine the N-terminal amino acid sequence, the purified protein fraction was applied to a protein sequencer PPSQ-10 (Shimadzu, Kyoto, Japan). For deglycosylation, purified tomato lectin was treated with trifluoromethanesulfonic acid (TFMS), by following the described method.[13] Molecular mass of the intact and deglycosylated tomato lectins were measured using a matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometer Voyger DE-Pro (Applied Biosystems). Sinapinic acid (3,5-dimethoxy-4- hydroxycinnamic acid) was used as the matrix.

Hemagglutination assay. Hemagglutinin activity was assayed as reported previously.[13] The activity was titrated by serially diluting the sample with PBS and then mixing it with an equal volume of a 2% suspension of rabbit erythrocytes. If necessary, *N,N',N"*-tri- acetylchitotriose, which was purchased from Seikagaku-kogyo (Tokyo, Japan), was used as a competitor.

Cloning of genomic tomato lectin gene. A set of oligonucleotide primers (5′-TAAGTGTTTTATGTGTGGTGAC-3′, 5′-TCAATCACTTGACGAACAGTCC-3′) was designed based on the nucleotide sequence of two EST clones; BP876111 and AJ784504, in order to amplify a part of the tomato lectin gene (Fig. 1) using the genomic DNA of Momotaro-Yoku as the template. The PCR product (*ca*. 1 kb) was integrated into pGEM-T Easy plasmid vector (Promega, Madison, WI) and the nucleotide sequence was determined. The cloned fragment was labeled with digoxigenin and used as the probe for Southern hybridization against restriction fragments of the Momotaro-Yoku genome, by following the manufacturer's instructions (Roche Diagnostics, Basel, Switzerland). The *Sau*3AI fragments containing the *ca*. 1.8 kb hybridized fragment were self-ligated with T4 DNA ligase and applied for inverse PCR as the templates using specific primers (5′- TCTCATCCGTCATGAATCCA-3′, 5′-CTATCGGCTGAGACAAGGAA-3′) that were designed in this study, based on the nucleotide sequence of the 1st PCR product (Fig. 1). The product of the inverse PCR with the expected size was cloned and sequenced. The whole gene and its flanking regions were finally amplified by PCR using a distinctive set of primers (5′-CGAAATTGATTTGGTTGTTA TG-3′, 5′-TCAATCACTTGACGAACAGTCC-3′) that were specifically designed based on the nucleotide sequence of the product of inverse PCR (Fig. 1). The PCR product with the expected size was cloned into pGEM-T Eazy. Sequence data was analyzed using Genetyx ver. 7 (Genetyx, Tokyo, Japan). The determined nucleotide sequence has been deposited in DDBJ with the accession number AB598780.

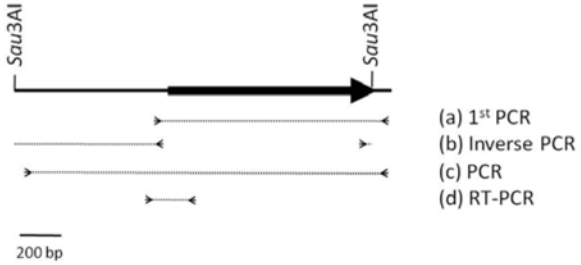

Fig. 1. Physical map of the lectin gene and its flanking regions. The thick line with arrow head shows the lectin gene. Small arrow heads and dotted lines indicate the primer positions and the target regions for first amplification, respectively (a), inverse PCR (b), amplification of the lectin gene and its flanking regions (c), and RT-PCR (d).

Transcriptional analysis of tomato lectin gene. DNA-free total RNAs were prepared from tomato fruits and other organs using an RNeazy Plant Mini Kit (Qiagen, Darmstadt, Germany) and an SV Total RNA Isolation System (Promega, Madison, WI) by following the manufacturers' instructions. A primer set (5′-AATGAAGGAGACCTTAATTA-3′, 5′-GC GGAGCACAATAACTTTGT-3′) specific for tomato lectin gene was designed to give PCR product of 257 bp (Fig. 1). Reverse transcription-PCR (RT-PCR) was done using an AccuPower RT/PCR Premix (Bioneer, Daejeon, Korea) according to the manufacturer's instructions. For PCR, the number of cycles was set to 26, avoiding saturation of PCR product formation. RT-PCR experiments without prior RT were done to ensure that no residual DNA was present in the RNA samples. As controls, transcripts of the *ef1α* and *ubi3* genes were detected in Momotaro-Yoku and Micro-TOM, respectively, by following the literatures,[24, 26] using the corresponding primer sets (5′-ATGTTGGGTTCAATGTTAAG-3′ and 5′-ATC ACACTGACAGTTCAC-3′ for *ef1α*; 5′-AGAAGA AGACCTACACCAAGCC-3′ and 5′- TCCCAAGG GTTGTCACATACATC-3′ for *ubi3*).

RESULTS AND DISCUSSION

Purification and characterization of a lectin from Momotaro-Yoku

A protein exhibiting hemagglutinating activity was purified from fruits of a horticultural variety of large tomato, Momotaro-Yoku, by two consecutive cation-exchange column chromatographies, following a reported method[13] with some modifications. One mg of the protein was purified from 1 kg of tomato fruits. The apparent molecular mass of the purified protein was over 97 kDa on an SDS-PAG (Fig. 2) and the N-terminal amino acid sequence was determined to be MPLSS, which was identical with that of the cherry tomato lectin (CTL).[13] The hemagglutinating activity of the purified protein was inhibited by *N,N',N"*-tri-acetylchitotriose. The molecular masses of the purified protein and its deglycosylation forms were determined to be 63,355 and 32,765 Da, respectively, by MALDI- TOF/MS (data not shown). The obtained values corresponded well with those reported in the CTL.[16] The data also coincided with the fact that lectin from common tomato is composed of equal amounts of protein and carbohydrate.[12] It was thus demonstrated that Momotao-Yoku produced a lectin whose characteristics were very similar with those of other reported tomato lectins. The lectin was named LTL (large tomato lectin).

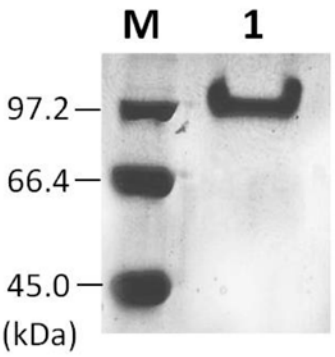

Fig. 2. Analyses of the purified tomato lectin by SDS-PAGE. Lane 1, Purified tomato lectin. Molecular size standards are shown in lane M and their molecular masses are indicated. Proteins were stained by CBB-R250.

Structure of tomato lectin gene of Momotaro-Yoku

A 1220-bp fragment was successfully amplified from the genomic DNA of Momotaro-Yoku using primers which were designed based on EST clones (see "Materials and methods" for details). The amino acid sequence deduced from the nucleotide sequence was completely identical to that of the corresponding part of CTL.[13] By inverse PCR and normal PCR, the whole part of the *LTL* gene and its flanking regions were obtained (Fig. 1). The DNA fragment obtained (1771 bp) contained a putative gene which encoded a protein composed of 365 amino acids. The nucleotide sequence of the *LTL* gene showed 99.8% (1093/1095 nt) identity to that of the *CTL* cDNA.[16] The length and the deduced amino acid sequence of the encoded protein LTL were identical to those deduced from the CTL cDNA. The nucleotide sequence of the 5′-end and upstream region of *LTL* exhibited striking identity to the contig sequence SlSBM_061361_01 in the tomato SBM (selected BAC clone mixture) data base (http://www.kazusa.or.jp/tomato/) at the Kazusa DNA Research Institute.

Expression of the lectin gene during tomato fruit ripening

As tomato lectins were mostly purified from fruits,[11-13,20] we first investigated the expression of the *LTL* gene in fruits of Momotaro-Yoku at distinctive ripening stages (Fig. 3). As controls, transcripts of the *ef1α* and *ubi3* genes were detected in Momotaro-Yoku and Micro-TOM, respectively, by following the literatures.[24, 26] Fruits of a model tomato variety Micro-TOM were also subjected to the investigation, to compare the expression pattern between large and mini tomato fruits. In Momotaro-Yoku, *LTL* transcription apparently increased from the immature green stage to the mature green/orange stage, whereas the amount of transcripts seemed decreased in red fruits. The pattern of the *LTL* transcripts amounts was similar to that of the ratio of *LTL*/*ef1α* transcripts. In immuno-blot analysis, tomato lectin was detected a little in immature green and matured red fruits, and the amount seemed rather higher in matured red fruits than in immature green ones. Intensive level of lectin was detected in matured green/orange fruits. The data suggest that *LTL* transcription was upregulated and the corresponding protein LTL was accumulated during the initial ripening stage. The accumulated lectin might be degraded in matured fruits. Alternatively, the lectin protein in matured fruits might be almost undetactable by the *anti*-CTL ntiserum, possibly because of a stage-specific modification or processing of LTL. In Micro-TOM, the transcripts were scarcely detected in immature green and mature green fruits, whereas strong transcription was detected in orange fruits while the level decreased in matured red fruits. The transcription pattern of the lectin gene in Micro-TOM, thus, seemed distinctive from that in Momotaro-Yoku, although the amount of transcripts peaked in orange fruits of the both varieties. The mRNA and the product protein of the lectin gene were investigated in the leaf, stem, and root of Micro-TOM to observe the organ specificity of gene expression. The transcripts and protein were detected only in fruits, suggesting that expression was fruit-specific (Fig. 4). The transcription of the lectin gene seemed higher in locular fluid of tomato fruits than in the pericarp of Micro-TOM (Fig. 4).

In this report, we clarified the structure of the lectin gene in the Japanese large tomato cultivar Momotaro-Yoku, so that transcriptional analysis was able to be accomplished. Transcription of the tomato lectin gene was shown to be organ- and stage-specific (Fig. 3 and 4). In spite of the fruit ripening-dependent manner of the transcription, binding sites for the tomato fruit-ripening regulator RIN[4] were not found upstream of the *LTL* gene.

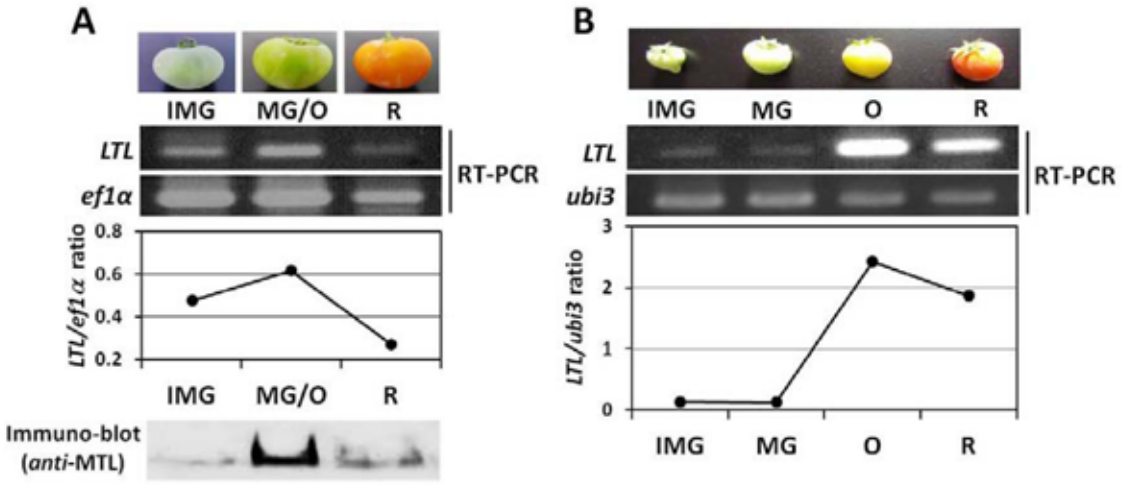

Fig. 3. Expression of the lectin gene in fruits of Momotaro-Yoku (A) and Micro-TOM (B).

Fruits at immature green (IMG), mature green (MG), orange (O) and red (Red) stages, which are shown on top, were taken 40 days after flowering and used for RNA and protein preparation. The expression levels were investigated by reverse transcription (RT)-PCR and immuno-blot analyses. For RT-PCR, 0.25 μg total RNA was used. The transcripts of the *ef1α* and *ubi3* genes are shown as controls in Momotaro-Yoku and Micro-TOM, respectively. For immuno-blot analysis 4 μg proteins were electrophoresed and tomato lectin was detected using *anti*-cherry tomato lectin (CTL) antiserum. The ratio between transcript amounts of *LTL* and *ef1α*/*ubi3* are also shown. The experiment was performed twice using two independent sets of tomato fruits composed of different developing stages and the result of one of the experiments is shown here as the representative.

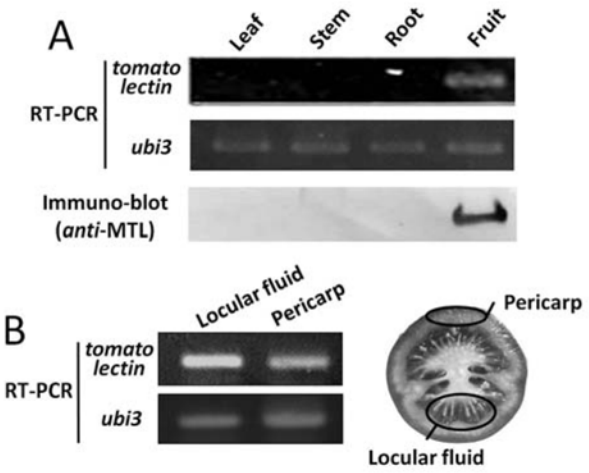

Fig. 4. Expression of the lectin gene in leaves, stems, roots and fruits of Micro-TOM.

(A) Leaves, stems, roots, and fruits were sampled 40 days after seeding. 0.25 μg total RNA and 5 μg protein were subjected to RT-PCR and immuno-blot analysis, respectively. The *ubi3* transcripts are shown as the RT-PCR control. Immuno-blot analysis was done using the *anti*-cherry tomato lectin antiserum. (B) Transcripts of the lectin gene in pericarp and locular part of fruits. 0.25 μg RNAs, which were prepared from those parts, were subjected to RT-PCR. Pericarp and locular parts are shown.

Cherry tomato lectin (CTL) binds high-mannose type *N*-glycans produced by plants and yeast.[15] High-mannnose type free *N*-glycans increase significantly in accordance with fruit ripening of tomato.[14] Free *N*-glycans in the pericarp account for >1 μg/g of the fresh weight of tomato, which further increases during the ripening process[18] and blocking of *N*-glycosylation with tunicamycin delays fruit ripening.[19] Because of the fruits ripening-specific expression of the lectin gene (Fig. 3 and 4) and the ability of CTL to bind *N*-glycan,[15] it could be assumed that tomato lectin is involved in fruit ripening and/or the following softening. Each of the CTL[16] and LTL (This study) contains a domain called extensin-like domain, which shares similar amino acid sequence with that of a cell wall protein extensin.[4] Structural feature of tomato lectins, which possessess a extensin-like domain flanked by four hevein-like putative sugar-binding domains,[18] may imply its biological roles as a cell wall protein. It is reported that banana (*Musa acuminate*) lectin gene is also expressed in ripening stage of fruits.[5] A gene for the putative tomato lectin Lycesca,[17] whose domain arrangement was distinctive from CTL and LTL, has been reported to be expressed in a stage-specific manner in developing tomato fruit.[21] Besides the proposed defensive role of plant lectins that have anti-fungal activities, some plant lectins might be involved in fruit ripening. Knock-down and high-expression experiments of lectin genes in tomato and other plants have remained to be conducted to elucidate the involvement of lectins in fruit ripening as well as in defensive processes. By elucidating the biological roles of the lectin in tomato plants, we may be able to obtain clues to develop novel technologies for enhancing the defensive ability of plants and/or controlling the maturation process of fruits of agriculture plants including tomato.

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