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Molecular cloning and functional expression of lewis type α1,3/α1,4-fucosyltransferase cDNAs from *Mangifera indica* L.

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ABSTRACT

In higher plants, complex type *N*-glycans contain characteristic carbohydrate moieties that are not found in mammals. In particular, the attachment of the lewis a (Le^a) epitope is currently the only known outer chain elongation that is present in plant *N*-glycans. Such a modification is of great interest in terms of the biological function of complex type *N*-glycans in plant species. However, little is known regarding the exact molecular basis underlying their Le^a expression. In the present study, we cloned two novel lewis type fucosyltransferases (*MiFUT13*) from mango fruit, *Mangifera indica* L., heterologously expressed the proteins and structurally and functionally characterized them. Using an HPLC-based assay, we demonstrated that the recombinant MiFUT13 proteins mediate the α1,4-fucosylation of acceptor tetrasaccharides with a strict preference for type I-based structure to type II. The results and other findings suggest that *MiFUT13s* are involved in the biosynthesis of Le^a containing glycoconjugates in mango fruits.

1. Introduction

Protein glycosylation is a universal post-translational modification that occurs in eukaryotic cells. *N*-linked oligosaccharides (*N*-glycans), which are attached to the asparagine residue specified by the sequence Asn-X-Ser/Thr, share a common trimannosyl core structure [Manα1,6(Manα1,3)Manβ1,4GlcNAcβ1,4GlcNAc], and it is widely accepted that their structural diversity depends on an intricate interplay between the processing enzymes. Based on our current understanding of the biosynthesis of *N*-glycans in higher plants, the initial steps that take place in the endoplasmic reticulum and in the early Golgi apparatus are similar to those for mammalian systems. However, subsequent maturation steps differ significantly between mammalian and plant cells because there are a number of functional differences in the *medial*/*trans*-Golgi compartments (Strasser, 2007). For example, because some plants lack glycosyltransferases that are involved in certain maturation and/or modification processes, such as further branching of β-linked *N*-acetylglucosamine (GlcNAc) residues, the assembly of a β1,4-linked bisecting GlcNAc, and the elongation of terminal β1,4-galactose and α2,3/α2,6-sialic acid residues (Bakker et al., 2001a; Zeleny et al., 2006), the resulting *N*-glycans are considerably less heterogeneous than those of mammalian *N*-glycans.

Plant *N*-glycans can be classified into two major subgroups, namely complex type and pauci-mannose type. Complex types generally contain one or two β1,2-linked GlcNAc residues in their outer chains. On the other hand, pauci-mannose types have a truncated structure resulting from the β-*N*-acetylhexosaminidase-mediated trimming of the complex type *N*-glycan (Liebminger et al., 2011). In spite of being differently processed, both types of *N*-glycans frequently contain unique β1,2-linked xylose and α1,3-linked fucose residues attached to the junction between the β1,4-mannose and the innermost GlcNAc of their core structure (Wilson and Altmann, 1998). The combination of such modifications is a characteristic feature, which is unique to plants but is not found in mammalian systems. It is noteworthy that *N*-glycans with β1,2-xylose and α1,3-fucose epitopes can elicit an undesirable immune response in humans, and are likely to constitute a major class of allergenic determinants on a variety of plant glycoproteins (Bardor et al., 2003; Jin et al., 2008).

In addition, a portion of plant *N*-glycans bear the lewis a (Le^a) trisaccharide structure [Galβ1,3(Fucα1,4)GlcNAc] at their non-reducing termini. It has been proposed that the assembly of the Le^a structure is the only known outer chain elongation of complex type *N*-glycans that takes place after the β1,2-xylose and α1,3-fucose residues are attached. During the last stage of *N*-glycosylation

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within the *trans*-Golgi, a β1,3-galactosyltransferase transfers a galactose to the non-reducing terminal β1,2-linked GlcNAcs, resulting in formation of elongated type I *N*-acetyllactosamine structures (Strasser et al., 2007a). The α1,3/α1,4-fucosyltransferase then catalyzes the transfer of a fucose to the GlcNAc residue of the disaccharide unit in an α1,4-linkage, thus completing the synthesis of the Le^a structure (Léonard et al., 2002). Unlike mammals, the Le^a epitope seems to be present exclusively on *N*-glycans (Strasser et al., 2007a), as reported for a number of soluble and membrane-bound extracellular glycoproteins (Melo et al., 1997; Fitchette et al., 1999). Moreover, a variety of complex type *N*-glycans that contain the Le^a structure have been observed in plant cell walls, thus being assumed to play some role in tissue cohesion (Fitchette et al., 1999).

The precise role of the Le^a structure in plant *N*-glycans is unknown, but is of great interest in terms of its possible involvement in various biological events, such as development, fertilization and biophylaxis. However, because the relevant enzymes have not been characterized in many plant species, detailed information regarding the biochemical basis underlying Le^a expression remains to be clarified. Currently, structural information of the genes encoding β1,3-galactosyltransferase enzymes (GALT1 or GALT15) and the α1,3/α1,4-fucosyltransferase (FUT13) enzyme are very limited and only available in a few model plants (Bakker et al., 2001b; Wilson, 2001; Wilson et al., 2001a; Strasser et al., 2007a). In this study, we report on the molecular cloning and enzymatic characterization of a novel FUT13 from mango, *Mangifera indica* L., one of the most extensively cultivated tropical fruits worldwide, to extend our understanding of the characteristic oligosaccharides in plants.

2. Results

2.1. Isolation of mango cDNAs encoding putative α1,3/α1,4-fucosyltransferase

To date, there are more than 95,000 nucleotide sequence entries in the GenBank for the mango. However, cDNA database resources available for protein glycosylation-related genes are scarce. To obtain genetic information for mango α1,3/α1,4-fucosyltransferase, a partial cDNA fragment was amplified using the RT-PCR method by referring to sequences that were identically conserved among the known plant *FUT13* genes. Based on the sequence information obtained from the product, the overall coding sequence was determined by amplification and sequencing of the unknown 5′ and 3′ terminal regions using RACE methods. As the result, a 1.2 kb length product was obtained by PCR amplification and was found to be a mixture of two different but apparently related candidates, as indicated by a 98% nucleotide sequence identity. Comparison of the sequences showed an insertion/deletion of twelve base lengths in their 5' terminal regions and eight nucleotide substitutions. Of these nucleotide substitutions, five were found to be synonymous and three were non-synonymous that resulted in changes in the amino acid sequences. Using Pfam and TMHMM online servers, both proteins encoded by these different candidates were predicted to be Golgi type II membrane proteins that can be classified as members of the glycosyltransferase family 10. Thus, we designated the two putative *FUT13* clones as *MiFUT13A* and *MiFUT13B*, respectively.

2.2. Primary structures of the proteins encoded by MiFUT13A and MiFUT13B

MiFUT13A (accession no. LC259301) encodes a protein of 410 amino acid residues with a calculated molecular weight of 46105.26 (Fig. 1). On the other hand, *MiFUT13B* (accession no. LC259302) encodes a highly homologous protein with a molecular weight of 45730.86, in which the region from Ser^{34} to Ile^{37} is absent and contains amino acid substitutions at positions corresponding to Gln^{40}, Arg^{75} and Val^{225} of the MiFUT13A (Fig. 2). A multiple alignment analysis showed that the MiFUT13A and MiFUT13B share 73–79% sequence identities with other plant lewis-type α1,3/α1,4-fucosyltransferases found in *Arabidopsis thaliana*, *Solanum lycopersicum*, and *Beta vulgaris* (Fig. 2). As shown by the comparison, it was found that the MiFUT13A and MiFUT13B proteins contain several conserved peptide sequences responsible for the catalytic activities and a C-terminal CXXC motif present in mammalian GT10 family α1,3-fucosyltransferases (Holmes et al., 2000). In particular, the sequences that are highly identical with the α3 motif V, which has been well characterized as a cluster of the key residues responsible for donor GDP-fucose and acceptor oligosaccharide binding, are conserved (Mollicone et al., 2009) (Fig. 3C). The alignment also suggests that MiFUT13A and MiFUT13B possesses possible acceptor binding motifs and another catalytic motif, designated as the 1st cluster motif that consists of $S(N/H/D)X_{5\text{-}9}RX_{5\text{-}7}(V/L/I)\text{-}X_3G$ located on the upstream of motif V (Both et al., 2011) (Fig. 3A and B). In addition, predictions using hydrophobic cluster analysis revealed the presence of slightly modified versions of the DxD motif, DLE, except that AtFUT13 contains ELE, a sequence that binds a divalent ion that assists in anchoring the pyrophosphoryl group of the donor sugar-nucleotides in the active site (Wiggins and Munro, 1998) (Fig. 4). As shown, while Wilson et al. predicted that KDD or EDD located in the hydrophobic pockets might be involved in the binding of divalent cations (Wilson et al., 2001a), this hypothesis prompts the question as to whether the positively charged ε amino group of the lysine residue might impair the interaction between the enzyme and manganese ion via repulsive electrostatic interactions. If the pKa value of the amino group were to be sufficiently low, the group would be in an unprotonated form, which would not be expected to exert such an unfavorable action but could serve as a ligand that forms a coordinate bond. However, a protonated form seems more likely due to an electrostatic stabilizing effect by the negatively charged carboxylic group of the neighboring aspartate residue, which must be deprotonated in order to form a coordinate bond. Therefore, it may be concluded that DLE is likely involved in the donor-binding of the MiFUT13 proteins although further investigation of this will be needed.

2.3. Heterologous expression of the recombinant MiFUT13 proteins in COS1 cells

In order to verify and characterize the enzymatic activities of the proteins encoded by *MiFUT13A* and *MiFUT13B*, we constructed heterologous expression systems using a COS1 cell line. The clonal cell lines stably transformed with the expression plasmid for a fused form of MiFUT13 with a C-terminal hexahistidine tag were established and were designated as COS1/MiFUT13A and COS1/MiFUT13B. For a negative control, the cell line transformed with the empty vector was prepared and designated as COS1/Mock. By end point RT-PCR, a significant expression of MiFUT13 mRNAs was detected in both the COS1/MiFUT13A and COS1/MiFUT13B cell lines (Fig. 5C). Furthermore, the expression of the recombinant MiFUT13 proteins was confirmed by western blot analysis using whole cellular lysates and anti-tetrahistidine antibodies (Fig. 5D).

2.4. Enzymatic characterization of recombinant MiFUT13 proteins

To investigate the enzymatic properties of the recombinant MiFUT13A and MiFUT13B proteins, their catalytic activities were assessed by HPLC-based assays using various acceptor oligosaccharides. Whole lysates prepared from COS1/MiFUT13A and COS1/