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# Expression of Human α-Lactalbumin in Transgenic Tobacco

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**α-Lactalbumin (αLA), a major milk protein, is the regulatory subunit of lactose synthase. To assess the production of recombinant αLA in plants, the cDNAs for human αLA with or without its own signal sequence were introduced into tobacco plants under the control of the cauliflower mosaic virus 35S promoter. The gene integration and expression at the mRNA level were confirmed in several regenerated plants, while the expression at the protein level could be confirmed only in a transgenic tobacco transformed with the gene containing the signal sequence. The tobacco-expressed αLA migrated in SDS-PAGE with identical mobility to αLA prepared from human milk, indicating that the signal peptide of human αLA was correctly processed to yield a mature protein in tobacco plants. The expressed αLA (*ca.* 5 μg/g of fresh leaves) was found in the soluble fraction and eluted from a DEAE-Sepharose column in the same salt concentration range as the milk αLA. The partially purified tobacco-αLA was fully active in the synthesis of lactose when combined with galactosyltransferase. Thus, the transgenic tobacco produces a fully active mature αLA in a soluble form.**

**Key words: folding, α-lactalbumin, nutrition, signal peptide, transgenic tobacco.**

α-Lactalbumin (αLA) is a major milk protein and in the mammary gland regulates the biosynthesis of lactose (for reviews, see Refs. *1-3*). αLA is synthesized only in the lactating mammary gland, where it combines with the Golgi-associated β-1,4-galactosyltransferase [EC 2.4.1.38] to form the lactose synthase [EC 2.4.1.22] complex. αLA acts as a $K_m$ modifier of the galactosyl acceptor, lowering the $K_m$ for glucose from above 1 M to the millimolar range, which allows for the synthesis of lactose under physiological conditions. The primary and tertiary structures of αLA are known to be homologous to the C-type lysozymes such as hen egg-white lysozyme, although the two proteins are different in function and localization (*3*). These features have led to extensive studies of αLA.

Since the recombinant αLAs cloned and expressed in *Escherichia coli* are reported to accumulate as undesirable inclusion bodies (*4, 5*), we are interested in testing a plant expression system for this protein. Although to date many proteins from various origins have been expressed in transgenic plants (*6*), further examples of mammalian proteins are desirable. In addition to providing an efficient expression system for recombinant proteins, successful production of αLA by plants would eventually lead to nutritional improvement of crops (*7*), as the amino acid composition of αLA is ideal from the nutritional standpoint (*8*). It is also possible that αLA produced in plant cells might act on the plant galactosyltransferase to synthesize lactose or inhibit glycoprotein synthesis. The activation of lactose synthesis by the addition of bovine αLA to galactosyltransferase in the Golgi membrane of onion stem has been reported by Powell and Brew (*9*). The introduction of a modifier protein to regulate the plant's own enzyme activity is a new approach to engineering plants. In the present study we have tested the expression of human αLA in tobacco plants.

## MATERIALS AND METHODS

*Materials*—The El2ΩGUSN/PBI vector, which is a modified pBI121 (CLONTECH) having a double enhancer and the Ω sequence of tobacco mosaic virus on both sides of the constitutive cauliflower mosaic virus (CaMV) 35S promoter, was provided by Dr. Y. Ohashi of our institute. Bovine αLA was a gift from Dr. K. Nitta of Hokkaido University. Human αLA, anti-human αLA, and bovine galactosyltransferase were purchased from Sigma. The concentrations of bovine and human αLA were determined using $A_{280}(1\%) = 20.1$ (*10*) and 15.0 (*11*), respectively. The protein molecular weight markers were from Pharmacia and Amersham.

*cDNA Cloning and Vector Construction*—DNA was manipulated essentially as described previously (*12, 13*). For PCR amplification of the human αLA gene from a human mammary gland cDNA library, the primers were designed to add a *Bam*HI and *Sac*I site (plus a 4-base clamp) to the ends of the PCR products. Thus, the "ATG primers" begin with 5′-GTCA**GGATCC**ATG (*Bam*HI site in bold) followed by either the N-terminal coding sequence for the signal peptide or for the mature protein except the N-terminal residue (total 30 bases). The latter primer

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Abbreviations: αLA, α-lactalbumin; *p*-APMSF, (*p*-amidinophenyl)-methanesulfonyl fluoride hydrochloride; CaMV, cauliflower mosaic virus; RT, reverse transcription.

would result in N-terminal Met in place of native Lys. The "TGA primer" begins with 5′-CGTA**GAGCTC**TCA (*Sac*I site in bold) followed by the anti-sense C-terminal sequence (total 30 bases). PCR was performed on QUICK-Clone Human Mammary Gland cDNA (CLONTECH) with *Pfu* DNA polymerase (STRATAGENE) for 30 cycles, yielding two PCR products depending on the primer set used: αLA with its signal sequence (αLA+S) and without (αLA−S). The PCR products were gel-purified using a MERMAID kit (BIO 101), digested with *Bam*HI and *Sac*I, and ligated to Bluescript II KS− (STRATAGENE) to transform *E. coli* XL1-Blue. The αLA inserts, verified to have the correct sequence by DNA sequencing, were excised from the plasmids with *Bam*HI and *Sac*I and ligated to El2ΩN/PBI, which was obtained by removing the GUS (β-glucuronidase) gene from El2ΩGUSN/PBI after digestion with *Bam*HI and *Sac*I. The resulting αLA expression plasmid El2ΩαLAN/PBI was first introduced into *E. coli* JM109, then into *Agrobacterium tumefaciens* LBA4404 by electroporation. The transformation was confirmed by PCR using the 35S and NOS primers, which are 22-base oligodeoxynucleotides corresponding to certain regions of the 35S promoter (sense) and the NOS terminator (anti-sense), respectively.

*Transformation of Tobacco*—Tobacco (*Nicotiana tabacum* cv. Petit Havanna SR1) leaf was transformed with El2ΩαLAN/PBI according to the method described by Horsch *et al.* (*14*), using the *Agrobacterium* binary vector system (*15*). Leaf pieces were briefly soaked in a culture of *A. tumefaciens* harboring El2ΩαLAN/PBI and placed on Murashige and Skoog MS medium plates. A few days later the leaf tissues were transferred onto media containing 0.2 mg/liter 1-naphthaleneacetic acid, 1 mg/liter 6-benzylaminopurine, 100 mg/liter kanamycin, and 500 mg/liter claforan. Shoots that developed were regenerated on the media without the hormones. The kanamycin-resistant plants were transferred to soil and grown in the greenhouse.

*PCR*—An ASTEC thermal cycler PC-700 was used for all PCR experiments. PCR for cloning was performed using *Pfu* DNA polymerase by the same method as used previously (*13*). For analysis and DNA sequencing, *Taq* DNA polymerase (Perkin Elmer) was used. Tobacco leaf DNA was extracted according to the method of Wang *et al.* (*16*). mRNA was prepared from young leaves using a QuickPrep Micro mRNA Purification kit (Pharmacia), and cDNA for reverse transcription (RT)-PCR was synthesized using a First-Strand cDNA Synthesis kit (Pharmacia).

*DNA Sequencing*—A Perkin Elmer-Applied Biosystems 373A DNA sequencer was used in combination with the *Taq* DyeDeoxy Terminator Cycle Sequencing kit. The plasmid DNA was prepared by a modified alkaline lysis method that involved treatment twice with 7.5 M ammonium acetate instead of sodium acetate as in the original protocol (*17*), and purified by precipitation with polyethylene glycol (*17*). For direct sequencing of PCR products, the amplified DNA fragments were precipitated with ammonium acetate and isopropanol to remove PCR primers and deoxyribonucleotides (*18*). In the cycle sequencing reactions, the manufacturer's instructions were modified so as to use 0.15–0.3 pmol of PCR product and 10–20 pmol of sequencing primer.

*Protein Extraction and Analysis*—Proteins were extracted by two methods: (i) Extraction with SDS-PAGE sample buffer containing 2-mercaptoethanol (SDS buffer). About 60 mg of fresh leaves in a 1.5-ml plastic tube, supplemented with 2 μl of 20 mM *p*-APMSF (Wako) and 120 μl of 1.5×SDS buffer, were ground on ice with a micro-pestle, boiled for 5 min, and centrifuged to remove debris. Then 2.5 μl of the supernatant was diluted to 10 μl with 1×SDS buffer and loaded onto the SDS-PAGE gel. About 10 mg of seeds were extracted with 200 μl of 1×SDS buffer containing 0.2 mM *p*-APMSF and loaded onto the gel in the same way. (ii) Extraction without SDS. About 80 mg of fresh leaves, supplemented with 1 μl of 20 mM *p*-APMSF and 1 μl of 2 M sodium phosphate buffer (pH 7 at 30 mM), were ground on ice as (i). Then 1 μl of the debris-free extract in a total of 10 μl of SDS buffer was loaded onto the SDS-PAGE gel after boiling for 5 min. About 15 mg of seeds were extracted with 60 μl of deionized water containing 0.5 mM *p*-APMSF. A mixture of 5 μl of the extract and 5 μl of 2×SDS buffer was loaded onto the gel.

SDS-PAGE was performed according to Laemmli (*19*), with 15% gel in 10×10-cm plates. Western blotting was done using a semi-dry electroblotting apparatus. Proteins were blotted on a Immobilon-P$^{SQ}$ membrane (Millipore). αLA bands were visualized with anti-human αLA (developed in rabbit) with the aid of a Vectastain Elite ABC kit.

The total amount of proteins in extracts was estimated using the Bio-Rad Protein Assay kit with bovine serum albumin (Sigma) as a standard.

*Purification of αLA*—The procedures used for milk αLA (*20*) were followed. To about 50 g of fresh leaves, 50 ml of 50 mM Tris-HCl (pH 7.8) containing fresh 0.1 mM Pefabloc SC (Merck) was added and the leaves were ground with a pestle in a mortar on ice. The supernatant of the centrifuged materials was fractionated with 43–75% saturated ammonium sulfate. The fraction was desalted with Amicon (PM10) and loaded onto a column (2.5×17 cm) of DEAE-Sepharose FF equilibrated with 20 mM Tris-HCl (pH 7.8). αLA was eluted with 0.25 M NaCl buffer. The αLA fractions were located by Western blotting. The partially purified αLA was desalted with Amicon and subjected to a second DEAE-Sepharose column (0.7×4.4 cm) chromatography with a gradient elution (NaCl from 0 to 0.25 M). The αLA fractions were pooled, concentrated, and desalted with Amicon.

*Assay of αLA*—The modifier activity of αLA was measured by its effect on the lactose synthase reaction: UDP-galactose+glucose→lactose+UDP. The spectrophotometric assay for αLA described by Fitzgerald *et al.* (*21*) was used, which measures UDP formation enzymatically, monitoring the reaction as a decrease in $A_{340}$ of NADH with time. Bovine galactosyltransferase was used as the catalytic component. For each assay, calibration curves with human or bovine αLA were made and the endogenous lactose synthase activity ($LS_{end}$) was also determined. Activity units are in μmol UDP formed/min.

## RESULTS

*cDNA Cloning of Human αLA*—Human αLA cDNAs were amplified from a human mammary gland cDNA library by PCR with the ATG and TGA primers containing *Bam*HI and *Sac*I sites, respectively as described in "MATERALS AND METHODS." Single major bands of the

expected sizes were observed in 2% agarose gel (data not shown): 449 bp was expected for αLA+S and 392 bp for αLA−S. The fragments were cloned into Bluescript II KS−. When the entire coding regions were sequenced, two independent clones gave the same sequence, which exactly matched the sequence reported in the literature (*22*).

*Transformation of Tobacco*—Tobacco leaf pieces were infected with *A. tumefaciens* carrying E12Ωα LAN/PBI. Transformed plants were selected on kanamycin. Shoots developed in about two weeks. A total of 31 regenerated shoots were transferred to hormone-free MS medium containing kanamycin for rooting. A total of 8 individual plants (4 each of αLA−S and αLA+S) survived and most of these were transferred to soil and grown to maturity, yielding seeds.

DNA was extracted from mature leaves of the regenerated plants (primary transformants) and the gene insertion was checked by PCR. Two primer sets (ATG+TGA primers; 35S+NOS primers) were used to confirm correct insertion of the expression cassette. The results with the 35S+NOS primers are shown in Fig. 1a. Two transformants with αLA−S (A1-5; A3-1) and three with αLA+S (B1-3; B2-2; B3-1) gave thick single bands of the expected sizes as in the vector constructs. When probed with the ATG and TGA primers, these transformants gave the same bands as those from the cDNA library (data not shown). The other transformants and untransformed control tobacco gave no corresponding bands in either PCR. We then conducted DNA sequencing of the PCR products obtained with 35S+NOS primers for A1-5, A3-1, B1-3, and B2-2 and confirmed the complete sequence of the tobacco-inserted αLA genes as well as part of the flanking sequences to be unchanged from those in the vector constructs.

*Expression of Human αLA in Tobacco*—Gene expression at the mRNA level was analyzed by RT-PCR of mRNA extracts from mature young leaves (Fig. 1b). All the transgenic plants that had the correct gene insertion as confirmed above gave bands of the expected sizes, demonstrating that they produce mRNA transcripts of the corresponding genes.

Gene expression at the protein level was analyzed by Western immunoblotting. Mature leaves were either extracted with SDS-PAGE sample buffer containing 2-mercaptoethanol or just ground by pestle to squeeze out the juice as described in "MATERIALS AND METHODS." These extracts, appropriately diluted to prevent band distortion, were subjected to 15% SDS-PAGE. Figure 2a shows the immunoblot with anti-human αLA. Only B2-2 (a transformant with αLA+S) gave a band at the same position as the standard human milk αLA. A more precise comparison with the milk αLA is shown in Fig. 2b. The results show that B2-2 produces αLA having the same molecular weight as that in human milk, which suggests that the signal peptide of the human milk protein was correctly processed to yield a mature protein in tobacco plants. Use of SDS-PAGE sample buffer for extraction did not improve the yields of extracted αLA, indicating that most of αLA is present in a water-soluble form in tobacco cells. The average content of αLA in fresh leaf tissue was estimated to be *ca.* 5 μg/g leaf, equivalent to *ca.* 0.2% of total soluble protein. We failed to detect expression of αLA in self-pollinated progeny seeds of B2-2 in a similar manner.

*Partial Purification of Tobacco αLA*—To further characterize the tobacco-expressed αLA, we tried to purify the protein from leaf extracts. About 50 g of B2-2 leaves kept

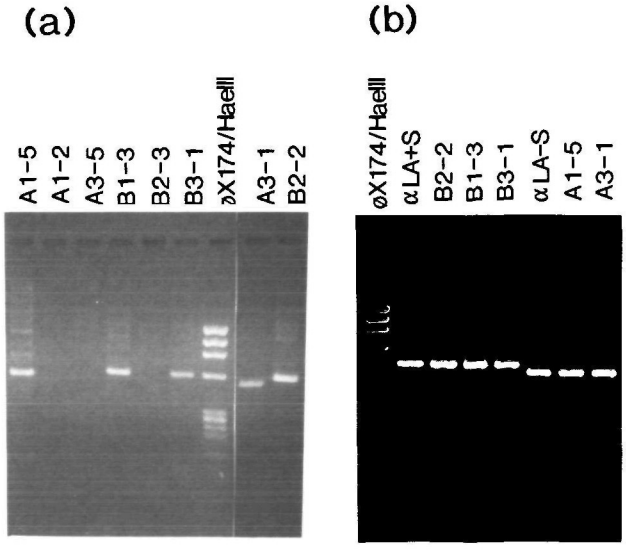


Fig. 1. **(a) PCR of leaf DNA of transformed tobacco.** The 35S and NOS primers were used. The PCR products were analyzed on 2% agarose gel stained with ethidium bromide. Transformants are denoted by A# (derived from the αLA−S gene) and B# (from the αLA+S gene). **(b) RT-PCR of leaf mRNA of transgenic tobacco.** Only transformants with the correct gene insertion were analyzed. The first-strand cDNAs synthesized from mRNA were subjected to PCR with the ATG and TGA primers. The effect of contaminating DNA in the present mRNA preparations was negligible, giving no corresponding bands by PCR (data not shown). αLA+S and αLA−S are the PCR products from mammary gland cDNA library.

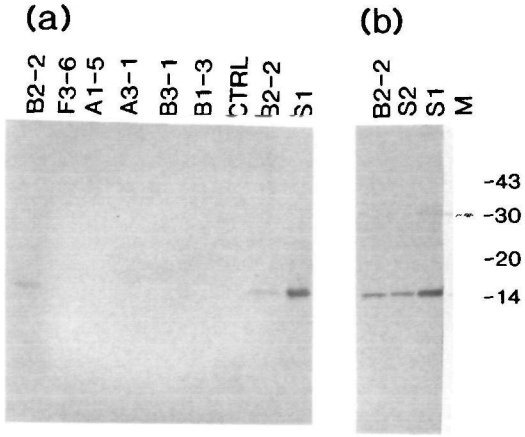


Fig. 2. **Western blot analysis of tobacco leaf extracts.** Proteins were separated by SDS-PAGE (15% gel), blotted on PVDF membrane, and probed with anti-human αLA. (a) The extracts were obtained from fresh leaves without addition of SDS. Transformants are labeled as A#, B#, and F#. F3-6 is a transformant unrelated to the present work. CTRL, untransformed tobacco; S1, standard human αLA from milk (42 ng). (b) αLA partially purified from B2-2 leaf extract (see text and Fig. 3) was compared with standard human αLA (S1=42 ng; S2=10 ng). Loading of varying amounts allowed estimation of the concentration of αLA in the partially purified sample (data not shown). M, marker proteins (Pharmacia) (stained separately with Coomassie Brilliant Blue). The molecular weights are indicated on the right in kDa.

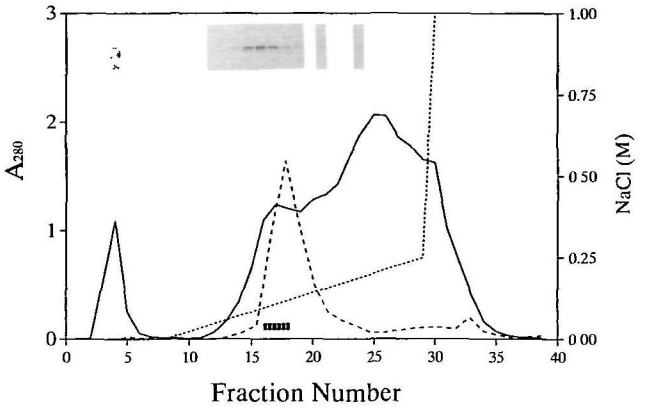


Fig. 3. **DEAE-Sepharose column (0.7×4.4 cm) chromatography of B2-2 leaf extract (—) and standard human αLA (----).** 1 fraction = 0.83 ml. For leaf extract, a fraction containing αLA from the first DEAE-Sepharose column was applied to a new column equilibrated with 20 mM Tris-HCl (pH 7.8) and eluted with NaCl gradient (·····). Immunoblots probed with anti-human αLA of selected fractions (performed as Fig. 2) are also shown above the elution profile. The fractions pooled for further analyses are indicated by ||||||. Standard sample was applied after regeneration of the column and eluted in the same way (plotted as $A_{280} \times 10$).

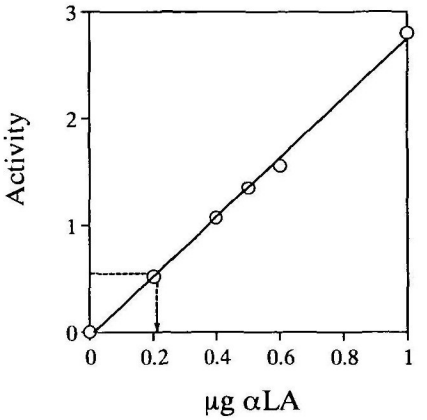


Fig. 4. **αLA assay.** Lactose synthase activity (μmol/min/ml enzyme stock solution) is plotted in $LS_{\alpha LA}$, which was obtained by subtracting $LS_{end}$. Assay mixtures contained various amounts of standard bovine αLA or leaf sample in a total volume of 1 ml. The amount of αLA in the partially purified tobacco αLA sample was evaluated as indicated by arrow.

at −80°C were used and processed as described in "MATERIALS AND METHODS." The fractions from the second DEAE-Sepharose column were analyzed by Western blotting. The elution profile of standard human milk αLA from the same column was also determined after the leaf sample. The results are shown in Fig. 3. It is apparent that two successive passages through DEAE-Sepharose columns were not enough to purify the tobacco αLA. It appeared that a significant loss of αLA occurred during ammonium sulfate fractionation or Amicon concentration, and we obtained only about 5 μg of αLA in the final Amicon concentrate. The SDS-PAGE analysis of the fractions failed to identify the αLA band when stained with Coomassie Brilliant Blue (data not shown). Nevertheless, the data in Fig. 3 show that the tobacco αLA eluted in the same NaCl concentration range as milk αLA, which suggests similar molecular charge properties for the αLAs from the two sources.

*Assay of Tobacco αLA*—The partially purified tobacco αLA was assayed for its ability to promote lactose synthesis. In the presence of a large quantity of galactosyltransferase used in the assay, the lactose synthase activity was detected in the absence of added αLA: $LS_{end} = 0.47$ unit/ml of enzyme stock solution (the values hereafter refer to a typical assay experiment). On the other hand, the tobacco αLA sample in the absence of galactosyltransferase caused a significant decrease in $A_{340}$ of the assay mixture with time (0.131/5 min), which is possibly due to contaminating substances that react with NADH. In the presence of galactosyltransferase, the slope was 0.226/5 min. The subtraction of the decrease in the absence of galactosyltransferase from this value gave a lactose synthase activity (LS) of 1.02 units/ml, which is significantly larger than the $LS_{end}$ value. Thus, the lactose synthase activity stimulated by αLA ($LS_{\alpha LA}$) is 0.55 unit/ml ($= LS - LS_{end}$). Calibration curves were made with milk αLA. An example of the assay with the bovine standard is shown in Fig. 4, from which the amount of αLA equivalent to bovine αLA was evaluated to be 42 μg/ml sample. Two other independent measurements were conducted with the tobacco sample and human milk αLA, which gave an average value of 47 μg/ml. These values roughly agree with that estimated from Western blotting (40 μg/ml; see legends to Fig. 2b) and show that the tobacco-produced αLA is fully active.

## DISCUSSION

In this study we have shown that the transgenic tobacco produces human αLA in a soluble form, which is indistinguishable from αLA in human milk with respect to its molecular size and biological function. This is notable when we recall that αLA is a small protein with molecular weight of *ca.* 14,100 yet possesses four disulfide bonds, which may make its spontaneous folding difficult and could be a cause of the formation of inclusion bodies in *E. coli*. It should also be noted that although lysozyme has been successfully expressed in transgenic tobacco (*23*), the conformational stability of αLA is much lower than that of lysozyme (*20, 24*).

The authentic signal peptide of human αLA was found to be functional and properly processed in tobacco plants. This result is consistent with other reports that the signal peptides of proteins from various sources, including bacteria and animals, were recognized and processed correctly in plants; thus, Sijmons *et al.* (*25*) reported that the pre-sequence, but not the pro-sequence, of human serum albumin was correctly processed in transgenic tobacco and potato plants. Human αLA is reported to contain no carbohydrate (*1*), although there is one possible *N*-glycosylation site in the sequence. The tobacco-produced αLA also appeared to be synthesized as a nonglycosylated form, as judged by its mobility in SDS-PAGE.

Human αLA, like bovine αLA, is claimed to have a well-balanced amino acid composition for the human diet (*8*). Its ingestion is considered to supplement the deficiencies of Lys and Met in cereals and legumes, respectively (*7*). Since αLA is a calcium-binding protein (*3*), it may also serve to supply calcium as a nutrient. The production of αLA in such crops by genetic engineering would, there-

fore, enhance the nutritional values of these crops (*26*). The present study opens the way to this approach. A current problem is that the transgenic tobacco produced only small amounts of αLA, but these are not exceptionally low as compared to those reported by other investigators for different proteins with the CaMV 35S promotor for constitutive expression. Although screening of a large number of transformants might have enabled us to find a transgenic line expressing up to 10 times as much as the present line, even this quantity would not be enough for the above purpose. The low production does not seem to arise from codon usage or signal peptide used, but may be inherent for the 35S promotor. Shade *et al.* (*27*) reported that the use of the promoter of the bean phytohemagglutinin-L gene gave excellent expression [1.2% (w/w)] of an α-amylase inhibitor in tobacco and pea seeds. At present, tissue-specific expression like this would be a way to produce a large quantity of foreign proteins in plants. This approach may be borne in mind together with the modification of storage proteins by genetic engineering as means to improve nutritional values of plants (*28*).

We now consider possible physiological effects of the expression of αLA on plants. The transgenic tobacco generated in this study grew normally and showed no apparent phenotypic changes as compared to control plants. However, this could be due to the low level expression of αLA. The mass production of αLA in plant cells might affect the physiology of the plant, since αLA might interact with galactosyltransferase in the Golgi, resulting in the synthesis of lactose and a partial inhibition of the synthesis of the carbohydrate moiety of glycoproteins. Thus, it may provide a way to assess the roles of the carbohydrate moiety of glycoproteins in cellular functions and to produce hypo-glycosylated proteins for structure-function studies. On the other hand, lactose is exclusively found in mammals (*29, 30*) and its production in plants may provide some dietary applications. It should be noted that the introduction of a protein like αLA that interacts with a plant enzyme and regulates its activity is a new approach to engineering plants. This approach can only be substantiated by greatly increasing the production level of the protein in plants.

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