Variation in Its C-Terminal Amino Acids Determines Whether Endo-β-Mannanase Is Active or Inactive in Ripening Tomato Fruits of Different Cultivars

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Endo-β-mannanase cDNAs were cloned and characterized from ripening tomato (*Lycopersicon esculentum* Mill, cv Trust) fruit, which produces an active enzyme, and from the tomato cv Walter, which produces an inactive enzyme. There is a two-nucleotide deletion in the gene from tomato cv Walter, which results in a frame shift and the deletion of four amino acids at the C terminus of the full-length protein. Other cultivars that produce either active or inactive enzyme show the same absence or presence of the two-nucleotide deletion. The endo-β-mannanase enzyme protein was purified and characterized from ripe fruit to ensure that cDNA codes for the enzyme from fruit. Immunoblot analysis demonstrated that non-ripening mutants, which also fail to exhibit endo-β-mannanase activity, do so because they fail to express the protein. In a two-way genetic cross between tomato cvs Walter and Trust, all F1 progeny from both crosses produced fruit with active enzyme, suggesting that this form is dominant and homozygous in tomato cv Trust. Self-pollination of a plant from the heterozygous F1 generation yielded F2 plants that bear fruit with and without active enzyme at a ratio appropriate to Mendelian genetic segregation of alleles. Heterologous expression of the two endo-β-mannanase genes in *Escherichia coli* resulted in active enzyme being produced from cultures containing the tomato cv Trust gene and inactive enzyme being produced from those containing the tomato cv Walter gene. Site-directed mutagenesis was used to establish key elements in the C terminus of the endo-β-mannanase protein that are essential for full enzyme activity.

In addition to helping produce a fruit suitable for consumption, ripening-associated softening in tomato (*Lycopersicon esculentum* Mill.) fruit leads to an increased susceptibility to physical damage during harvest and/or pathogen attack during shipping and storage. This results in crop losses that are costly to producers and consumers alike. Hence, there has been a great deal of research conducted to elucidate the mechanisms involved in ripening-associated softening. Softening in fleshy fruits is caused by the dissolution of pectin in the middle lamella, which reduces cell adhesion (Wakabayashi, 2000), and also by the breakdown of the cell walls themselves. Cell wall breakdown is caused by the concerted action of a number of proteins/enzymes including, but not limited to, expansins, which dissociate the xyloglucan/cellulose network (Brummell et al., 1999); pectin methylesterase, which cleaves methylester groups from pectic polysaccharides; polygalacturonase, which hydrolyzes pectin; and β-galactosidase, which removes the galactan side-chains from rhamnogalacturonan I (Brummell and Harpst, 2001).

Glucosamannans, galactoglucomannans, and galactomannans are polysaccharides present in type I cell walls, which are thought to cross-link cellulose microfibrils in the same manner as xyloglucans, although to a lesser degree (Carpita and Gibeaut, 1993). Of these three types of mannans, the cell walls of tomato are known to contain glucosamannans (Tong and Gross, 1988; Seymour et al., 1990). Endo-β-mannanase is capable of hydrolyzing these polysaccharides, and the activity of this enzyme increases in the outer tissues of tomato fruits during ripening (Bewley et al., 2000). The enzyme is also active in ripe fruits of a number of other species such as watermelon (*Citrullus vulgaris*), cantaloupe (*Cucumis melo*), and peach and nectarine (*Prunus persica*) (Bourgault et al., 2001). It has been suggested that endo-β-mannanase plays a role in ripening-associated softening (Pressey, 1989; Bewley et al., 2000), a hypothesis supported to some degree by the fact that the fruits of several non-ripening tomato mutants that fail to soften exhibit very low or no enzyme activity. However, fruit of the tomato cv Walter, which exhibit no endo-β-mannanase activity during ripening, appear to ripen normally, with only a minor difference in texture in fully ripened fruit (Bewley et al., 2000).

The gene for endo-β-mannanase is present in tomato cv Walter, it is transcribed in the fruit, the message is translated, and the resultant full-length protein is localized in the cell wall, as in cultivars producing active enzyme (Banik et al., 2001). There are no previous reports of permanently inactive hydrodrolases being produced in plant tissues; the fact that this occurs in tomato cv Walter fruit is intriguing. There are several examples where there is temporary inactivity of enzymes after their synthesis, for exam-
ple the Suc-cleaving enzyme invertase is present in the vacuole and cell wall during tomato fruit development and ripening but is only active during the latter process. The increase in activity during ripening is attributable to posttranslational regulation, which increases the activation state of invertase rather than the amount of enzyme present. Activation appears to be achieved by alterations in the amount of a protein inhibitor thought to control this enzyme (Husain et al., 2001). By mixing fruit extracts from cultivars producing active and inactive endo-β-mannanase, it is clear that an inhibitor is not the cause of enzyme inactivity in tomato cv Walter (Banik et al., 2001). The purpose of this study was to determine why the enzyme produced in the fruit of tomato cv Walter is never active.

To our knowledge, there are no previous reports of the purification, cloning, and characterization of endo-β-mannanase from tomato fruit. We have characterized the genes for endo-β-mannanase from cultivars producing active and inactive enzyme and have demonstrated that there is a two-nucleotide deletion in the gene from cultivars producing inactive enzyme. This mutation causes a frame-shift, which results in an altered, truncated C terminus of the enzyme in comparison with the active form. We show, by crossing two cultivars that carry either the active or inactive form of the gene and by heterologous expression, that this mutation is the only cause for the inactivity of the enzyme.

RESULTS

Immunoblot Analysis of Non-Ripening Mutants of Tomato

The fruits of several non-ripening mutants of tomato do not exhibit endo-β-mannanase activity during ripening, whereas their corresponding wild-type cultivars produce active enzyme (Bewley et al., 2000). Immunoblot analysis (Fig. 1) shows that whereas endo-β-mannanase protein is produced in the fruits of tomato cvs Trust, Ailsa Craig wild type (wt), and Rutgers wt, which exhibit enzyme activity, the tomato cv Ailsa Craig ripening inhibitor (rin), non-ripening (nor) and tomato cv Rutgers alcobaca (alc), Never ripe (Nr) non-ripening mutants, which lack activity, do not express the protein. Thus, the lack of activity in the non-ripening mutants is attributable to a lack of enzyme production. However, fruits of the tomato cv Walter also do not produce active endo-β-mannanase but nevertheless produce the appropriate mRNA transcripts (Banik et al., 2001) and endo-β-mannanase protein during ripening (Fig. 1). Why the fruits of this cultivar produce an enzyme that is inactive is investigated here.

Purification, Cloning, and Characterization of Endo-β-Mannanase from Tomato Fruit

Endo-β-mannanase was purified to homogeneity from the outer regions of ripe fruit of the tomato cv Trust. The apparent molecular mass of the enzyme, 40 kD, is in agreement with the size of the protein detected on western blots (Fig. 1). The first 13 N-terminal amino acid residues of the mature, active enzyme were determined to be FSNNNFVYTDGTH.

The cDNAs encoding endo-β-mannanase from fruits of the tomato cvs Trust and Walter were obtained by reverse transcriptase-PCR and named L. esculentum endo-β-mannanase 4 (LeMAN4a), active enzyme from tomato cv Trust fruit (GenBank accession no. AY046588) and L. esculentum endo-β-mannanase 4 (LeMAN4i), inactive enzyme from tomato cv Walter fruit (GenBank accession no. AY046589).

The 1,420-bp cDNA from the tomato cv Trust contains an open reading frame of 399 amino acids including a signal peptide of 26 amino acids (Fig. 2, underlined). The 13 amino acid N-terminal sequence determined by peptide sequencing begins at amino acid 27. Therefore, the mature enzyme from the tomato cv Trust contains 373 amino acids, which results in a protein with an estimated molecular mass of 42,398 D and a predicted pI of 8.75. These data are in close agreement with experimentally determined values reported earlier (Bewley et al., 2000; Bourgault et al., 2001). The potential N-glycosylation site (Fig. 2, double underlined) in both LeMAN1 (Bewley et al., 1997) and LeMAN2 (Nonogaki et al., 2000) is absent from LeMAN4a, but the putative catalytic sites Glu-204 and Glu-318 (Fig. 2, asterisks) are conserved (Nonogaki et al., 2000; Hogg et al., 2001). The Trp at position 360 is also conserved and is thought to play a critical role in substrate binding (Hogg et al., 2001).
There is a 46% identity and 67% similarity in amino acid sequence of the mature protein between LeMAN4a and LeMAN1. A comparison of LeMAN4a with LeMAN2 shows that the identity is 51% whereas the similarity is 70%. This increased similarity can be attributed largely to a 28 amino acid segment, starting at residue 124, which is present in both LeMAN4a and LeMAN2 but not in LeMAN1. It is interesting that within this 28 amino acid segment, the homology between LeMAN4a and LeMAN2 is quite high (61% identity and 75% similarity) particularly because LeMAN2 is specifically expressed in seeds and LeMAN4 in fruit. As noted by Nonogaki et al. (2000) this region also shows a relatively high homology with that in fungal mannanases. In a simple protein-protein BLAST search of the GenBank database, the closest existing match (62% identity and 78% similarity) to LeMAN4a is an endo-β-mannanase cloned from germinated coffee (Coffeea arabica) seeds (GenBank accession no. AJ278996; Marraccini et al., 2001). The cDNA sequence for endo-β-mannanase from tomato cv Walter is identical to that of the tomato cv Trust enzyme except for a two-nucleotide deletion starting at bp 1,209 of the full-length transcript (Fig. 3). This deletion causes a frame shift and an altered C-terminal amino acid sequence compared with that of the tomato cv Trust enzyme. We then sought to determine whether this alteration is responsible for the lack of activity in tomato cv Walter.

After a two-way cross of the tomato cvs Trust and Walter, all F1 plants produced fruit that exhibit endo-β-mannanase activity regardless of which was the maternal parent. Thus, the gene for the active form of the enzyme is dominant. Genomic DNA was extracted from leaves of the F1 plants and used in genetic screening tests to confirm the presence of both LeMAN4a from tomato cv Trust and LeMAN4i from tomato cv Walter. Using primers specific for each form of the endo-β-mannanase gene, PCR was performed to confirm that plants in the F1 generation are heterozygous (Fig. 4). Using primers specific for the active enzyme gene, LeMAN4a (Fig. 4A, TP), plasmid DNA containing this gene yielded a band of 351 bp as expected. There were no bands in the absence of these primers for the inactive form, LeMAN4i (Fig. 4B, TP). The two-nucleotide deletion at bp 1,209 is responsible for the lack of activity in cultivars containing this mutation.
of template, nor in the presence of tomato cv Walter genomic DNA. A band was obtained when tomato cv Trust genomic DNA was used as template, as expected. All of the F1 progeny derived from tomato cv Trust maternal plants (e.g. T3 and T4) and tomato cv Walter maternal plants (e.g. W10 and W12) contain the \( \text{LeMAN4a} \) gene. Using PCR with primers specific for the gene for the inactive enzyme, \( \text{LeMAN4i} \) (Fig. 4B), no bands were obtained using plasmid DNA including \( \text{LeMAN4a} \), control without template, nor tomato cv Trust genomic DNA as template. A band was obtained using tomato cv Walter genomic DNA as template. All of the F1 progeny derived from tomato cv Trust maternal plants (T3 and T4) and tomato cv Walter maternal plants (W10 and W12) contain the \( \text{LeMAN4i} \) gene. These results confirm that all of the F1 progeny examined were heterozygous crosses of the tomato cvs Trust and Walter and they all produced fruit with active endo-\( \beta \)-mannanase.

After self-pollination of flowers on a heterozygous plant of the tomato cv Walter maternal F1 group, seeds were collected from the mature fruit. These fruit were expected to exhibit segregation in a ratio of approximately 3:1 with respect to active:inactive endo-\( \beta \)-mannanase alleles. Of 28 F2 plants tested, five did not exhibit activity because they were homozygous recessive, carrying two copies of the inactive form of the gene. Therefore, the resultant ratio of 23:5 or 4.6:1 is somewhat higher than expected under ideal conditions but within statistical error for a sample size of 28. Using PCR with the same two sets of primers that were employed in screening of the F1 generation, plants of the F2 generation were screened for the presence of \( \text{LeMAN4a} \) and \( \text{LeMAN4i} \) (Fig. 5).

Primers specific for \( \text{LeMAN4a} \) detected at least one copy of the gene for the active form of the enzyme in 23 of 28 plants (Fig. 5A), as in the tomato cv Trust parent genomic DNA. Primers specific for \( \text{LeMAN4i} \) detected at least one copy of the gene coding for the inactive form of the enzyme in 17 of the 28 F2 plants (Fig. 5B), but not in tomato cv Trust parent genomic DNA. Therefore, it is evident that plants 9, 15 to 22, 27, and 28 are all homozygous with respect to the \( \text{LeMAN4a} \) form of the gene; whereas plants 2, 10, 13, 24, and 25 are homozygous with respect to the \( \text{LeMAN4i} \) form. Fruits from the latter set of plants did not exhibit endo-\( \beta \)-mannanase activity, whereas all others, whether heterozygous (plants 1, 3–8, 11, 12, 14, 23, and 26) or homozygous with respect to \( \text{LeMAN4a} \), did exhibit activity.

**Expression of Recombinant Proteins in *Escherichia coli***

Endo-\( \beta \)-mannanase cDNAs from both tomato cv Trust and tomato cv Walter were cloned into the...
the MBP/endo-β-mannanase fusion protein was detected by western blotting (Fig. 6B). The antibody bound to a single band in the lane containing proteins from uninduced cells and in that of soluble proteins from induced cells. The antibody bound to multiple bands in the lanes containing proteins from crude cell lysates of induced cells, likely because of the presence of partially synthesized and/or degraded products. Thus, there was some expression of the fusion protein before the addition of IPTG, but this increased greatly after induction. The molecular mass of the single band observed in the western blot of the uninduced and soluble proteins was the same as the intensely stained fusion protein band in the Coomassie Blue-stained SDS-PAGE gel in Figure 6A.

Soluble extracts from *E. coli* cultures containing only the pMAL expression vector did not exhibit endo-β-mannanase activity when subjected to the enzyme-specific gel-diffusion assay (Fig. 6C). Those from the cultures containing the MBP-LeMAN4a fusion protein did display activity, whereas those from cultures containing MBP-LeMAN4i did not.

### Site-Directed Mutagenesis at the C Terminus of LeMAN4: Effects on Enzyme Activity

The Quick Change Site-Directed Mutagenesis System (Stratagene, La Jolla, CA) was used to create mutations at the carboxy terminus of the *E. coli*-expressed MBP/endo-β-mannanase fusion protein as detailed in Table I. These changes were made to determine specifically which amino acid or amino acids are key for full enzyme activity in the LeMAN4a form of the protein. The relative changes in activity in the various constructs show that there are a number of amino acids in the C terminus that are essential for full enzyme function (Fig. 7). The activities of the non-mutated tomato cv Trust and tomato cv Walter clones are also included on the graph for comparison.

Construct 1 (Fig. 7), MBP-LeMAN4i, which has the same C terminus as endo-β-mannanase from tomato cv Walter, exhibits no enzyme activity as expected, whereas construct 7, MBP-LeMAN4a, which has the same C terminus as endo-β-mannanase from tomato cv Trust, has very high activity. Construct 2 is identical to LeMAN4i, construct 1, except that Ser-396, which is present at this position in LeMAN4a; this change resulted in a small increase in activity compared with construct 1. This construct can also be considered to be LeMAN4a (construct 7) with the four C-terminal amino acids (SKLS) removed. From this viewpoint, activity was reduced to less than 10% of full activity of LeMAN4a. Construct 3 is identical to LeMAN4i, construct 1, except that Ser-396, which is present in the LeMAN4a form of the protein, has been added. This change also resulted in an increase in activity in comparison with LeMAN4i. When both
of these changes were made together (construct 4), an increase in activity was observed that was approximately additive of those for the individual changes (constructs 2 and 3). Construct 4 can be considered as LeMAN4a (construct 7) with the three C-terminal amino acids (KLS) removed, in which activity was reduced to about 30% of full activity. Construct 5 is equivalent to LeMAN4a (construct 7) with the two C-terminal amino acids (LS) removed; the resultant enzyme had only 17% of the activity of LeMAN4a. When the Leu at position 398 was restored, as in construct 6, full-enzyme activity was reestablished. Thus, removing the C-terminal Ser had no effect on enzyme activity. To investigate the importance of Arg-394, which is changed to an Ala in LeMAN4i, this same change was made in the otherwise full-length LeMAN4a enzyme. This resulted in only a small reduction in activity (construct 8) in comparison with LeMAN4a (construct 7).

DISCUSSION

The activity of the enzyme endo-β-mannanase increases in the outer tissues of tomato fruits during ripening (Bewley et al., 2000). The pH of the enzyme expressed in fruit (Pressey, 1989; Bourgault et al., 2001) is much higher than that of isozymes expressed in the seed before (LeMAN2; Nonogaki et al., 2000) and after (LeMAN1; Bewley et al., 1997) germination. Furthermore, the enzyme expressed in the fruit is tightly associated with the cell walls (Bewley et al., 2000; Bourgault et al., 2001), whereas the seed isozymes are readily soluble in low-salt buffer. Therefore, the enzyme expressed in the fruit is distinct from other characterized endo-β-mannanases in tomato, and as demonstrated here by N-terminal sequence analysis of the purified protein and by cDNA cloning, there is a specific gene for the enzyme expressed in the fruit.

Endo-β-mannanase may play a role in cell wall breakdown that leads to ripening-associated softening of fruit tissue (Pressey, 1989; Fischer and Bennett, 1991; Bewley et al., 2000). Although this relationship has not been established, a number of non-ripening mutants that exhibit reduced softening are also lacking in, or contain very low endo-β-mannanase activity (Bewley et al., 2000). Western-blot analysis shows that the enzyme protein for endo-β-mannanase is not expressed in the fruit of the non-ripening mutants tomato cvs Ailsa Craig rin, nor, Rutgers Nr, and alc, which are deficient in endo-β-mannanase activity. The reason for the lack of expression is likely to be different for each of these non-ripening mutants because the maturing process of each is defective in a unique way. For example, non-ripening in the Nr mutant is caused by a defect in a receptor for ethylene (Wilkinson et al., 1995), which results in an interruption in the signal transduction pathway that normally leads to the synthesis of transcription factors, which initiate the expression of ripening-associated genes. The rin mutant contains a defect in the expression of a MADS box protein, which is a transcription factor involved in the nonhormonal or developmental regulation of ripening-associated genes (Vrebalov et al., 2002). In each case, the mutation results in a lack of expression of the endo-β-mannanase protein. This is not so in tomato cv Walter, because the inactive enzyme protein is present in extracts from the fruit of this cultivar. In tomato cv Walter, the gene encoding endo-β-mannanase is present, it is transcribed in the fruit, the mRNA is translated, and the protein is properly lo-

![Figure 7](image.png)
calized to the cell wall as in cultivars expressing active enzyme (Banik et al., 2001). Therefore, the lack of endo-β-mannanase activity in tomato cv Walter is likely attributable to a defect, or mutation in the enzyme itself. To examine this possibility, the active enzyme was purified from the outer tissues of tomato cv Trust fruit and characterized, and the cDNA (LeMAN4) was cloned from the fruit of cultivars producing active and inactive forms of the enzyme.

The deduced amino acid sequence of the protein encoded by LeMAN4 has a high homology with other endo-β-mannanases from tomato seed, LeMAN1 (Bewley et al., 1997) and LeMAN2 (Nonogaki et al., 2000) and an even higher homology with the enzyme expressed in germinated coffee seeds (Marraccini et al., 2001). The nucleotide sequences of the LeMAN4 cDNAs from tomato cv Trust and tomato cv Walter are identical with the exception of a two-nucleotide deletion starting at bp 1,209 in the gene from tomato cv Walter, which causes a frame-shift resulting in a truncated C terminus in the enzyme. Thus, the LeMAN4a active endo-β-mannanase is longer by four amino acids, Ser-Lys-Leu-Ser, and the Arg at position 394 is an Ala in LeMAN4i, the inactive form of the enzyme. Two other cultivars of tomato, cvs Indian River and Heinz 1439, which do not exhibit endo-β-mannanase activity in their fruit, contain the LeMAN4i form of the gene, whereas all cultivars tested that produce active enzyme contain the LeMAN4a form of the gene. To determine whether the two-nucleotide deletion is the sole cause of the lack of activity in LeMAN4i, two strategies were employed. The first strategy was to cross the tomato cvs Trust and Walter, to test the fruit from the F1 and F2 generations for endo-β-mannanase activity, and to determine which form of the gene the progeny carry. The second strategy was to express the two cDNAs as fusion proteins in E. coli and to observe differences in activity.

Analysis of the genotypes and endo-β-mannanase activity in the fruits of the F1 and F2 progeny of the crosses between the tomato cv Trust and tomato cv Walter confirmed that the two-base deletion in LeMAN4i was solely responsible for the lack of activity in fruits from plants carrying only that form of the gene. In addition, when LeMAN4a and LeMAN4i were cloned into the pMAL fusion protein vector and expressed in E. coli, both constructs produced a fusion protein that was recognized by the antibody specific for endo-β-mannanase in western-blot analysis. However, only the construct containing LeMAN4a produced a protein that exhibited enzyme activity when analyzed by the gel-diffusion assay. This not only confirms that the cDNA is the gene for endo-β-mannanase, but also that the difference in the C termini encoded by LeMAN4a and LeMAN4i is the reason for the lack of activity in the LeMAN4i form of the enzyme.

Mutations in the C-terminal region of the MBP-LeMAN4 fusion protein resulted in products with varying amounts of endo-β-mannanase activity. When sequentially replacing in LeMAN4i (in which Ala-394 has been changed to Arg-394 as in LeMAN4a) the four amino acids present at the C terminus of LeMAN4a, it is obvious that Leu-398 is very important for full enzyme activity. Upon examination of the amino acid alignment of other endo-β-mannanase sequences from tomato, it is notable that Leu also appears in this location in LeMAN1 (Bewley et al., 1997) and LeMAN2 (Nonogaki et al., 2000). In these seed-specific isozymes, Leu is not the penultimate amino acid as in LeMAN4a, but it is in ManB, from coffee seed (Marraccini et al., 2001), which shows higher overall homology with LeMAN4a than do the tomato seed isozymes. This Leu is also conserved in an endo-β-mannanase cDNA cloned from germinated lettuce (Lactuca sativa) seed (J. Li and J.D. Bewley, unpublished data). Thus, of the C-terminal four amino acids in the enzyme, Leu-398 is key for full enzyme activity, perhaps for proper protein folding to allow for substrate binding and/or nucleophilic attack.

MATERIALS AND METHODS

Plant Material

All tomato (Lycopersicon esculentum Mill.) plants were pot-grown in soil in the greenhouse of the Department of Botany, University of Guelph, under natural and artificial light conditions. Plants were fertilized once a week with 20-20-20 fertilizer including micro-nutrients, at a concentration of 250 mg L^-1. Flowers were self-pollinated except where specified in genetic cross experiments. Fruits were collected at the stages of ripening detailed previously (Bewley et al., 2000). Seeds of tomato cvs Ailsa Craig wt, rin, nor; tomato cvs Rutgers wt, alc, Nr; and tomato cv Walter wt were obtained from The C.M. Rick Tomato Genetics Resource Center, University of California (Davis). Seeds of the tomato cv Trust were obtained from The Greenhouse Country Market (Brampton, ON, Canada). Seeds of tomato cv Heinz 1439 were kindly supplied by Dr. K. Peter Pauls (Department of Plant Agriculture, University of Guelph), and those of tomato cvs Manalucie, Indian River, Manapal and Homestead 24 by Dr. J.W. Scott (Institute of Food and Agricultural Sciences, University of Florida, Bradenton).

Enzyme Extraction and Immunoblot Analysis

Combined exocarp and outer pericarp tissue was removed to a depth of approximately 2 mm from ripe tomatoes of the following cultivars: Trust, Walter, Rutgers wt, Nr, alc, and Ailsa Craig wt, rin, nor. For the non-ripening mutants, “ripen” was considered the maximum stage of ripening reached before any visible signs of spoilage. Tissue was pulverized to a fine powder in the presence of liquid nitrogen and washed sea sand. McIlvaine (1921) buffer, pH 5 (0.2 mM Na2HPO4 and 0.1 mM citric acid at a 1:1 ratio), plus 0.5 mM NaCl was added in a ratio of five parts buffer to one part tissue (v/w) and followed by more grinding, and centrifugation at 14,000g for 5 min at 4°C to obtain the supernatant. This was analyzed for endo-β-mannanase activity using an enhanced gel-diffusion assay (Bourgault and Bewley, 2002). Protein concentration was determined using the BCA Protein Assay Reagent (Pierce, Rockford, IL), and for SDS-PAGE, 10 μg total protein was loaded per lane onto a 12% (w/v) polyacrylamide gel. Electrophoretically separated proteins were transferred to a nitrocellulose membrane (Towbin et al., 1979), which was washed, blocked with 5% (w/v) skim milk, and challenged with a 10^-5-fold dilution of an anti-endo-β-mannanase antibody raised in rabbits against the M1 isoform of the enzyme from germinated tomato seed (Nonogaki et al., 1995). Bands were made visible on x-ray film using a goat
Purification and Characterization of Endo-β-Mannanase from Tomato Fruit Tissue

Fifty grams of combined excocarp and outer pericarp tissue (to a depth of approximately 2 mm) from ripe tomatoes was ground to a fine powder as above. The tissue was first extracted with 250 mL of ice-cold 0.1 M HEPES-NaOH, pH 8.0, with centrifugation at 10,000g for 10 min. This buffer does not extract endo-β-mannanase from the tissue but does remove most of the soluble protein (Bourgault et al., 2001). The pellet was resuspended in 100 mL of McIlvaine buffer, pH 5.0, plus 100 mM NaCl. After a 10-min incubation on ice with vigorous agitation, the suspension was centrifuged at 10,000g for 10 min, the supernatant was decanted, and a 40-mL aliquot was precipitated successively on ice with ammonium sulfate. After dissolving each of the pellets in 1.5 mL of McIlvaine buffer, pH 5, most of the activity was present in the 40% to 60% fraction. The entire 1.5 mL was concentrated to 200 μL using a 5-kD molecular mass cut-off membrane (Amicon, Beverly, MA). The concentrate was applied to the acidic end of an 8 to 10 pH isoelectric focusing horizontal slab gel (Multiphor 2117, LKB, Uppsala) for separation at 2,000 V for 90 min, and 1-cm strips spanning the length of the gel were excised and extracted using water. All of the endo-β-mannanase activity was in the strip at pH 9.1, and this ran as a single band on an SDS-PAGE gel. A portion of the pure extract (80 μL) was subjected to 12% (w/v) polyacrylamide SDS-PAGE and transferred to a polyvinyldene difluoride membrane, and N-terminal peptide sequencing was conducted (Protein Service Laboratory, University of British Columbia, Vancouver).

Preparation of RNA, Reverse Transcriptase-PCR, and Subcloning of LeMAN4 cDNAs

One gram of combined excocarp/outer pericarp tissue from stage 4 to 5 fruits of tomato cv Trust and cv Walter was pulverized in liquid nitrogen and extracted with 10 mL of Tri-Reagent (Sigma-Aldrich, St. Louis). Poly(A+) RNA was obtained using the PolyATtract mRNA isolation system (Promega, Madison, WI). First-strand cDNA synthesis was carried out using Thermoscript RT (Invitrogen, Carlsbad, CA) reverse transcriptase with an oligo(dT)17-adaptor primer and an oligonucleotide (5’-ATGGCAT-TTGCCAGGTTTAACTCAAGAG-3’). Using an aliquot of the cDNA mix as template, PCR was then performed with the oligo(dT)17-adaptor primer and an oligonucleotide (508-fwd, 5’-ATGCGAT-GGAACATCTGAATGACTGCG-3’) based on a highly conserved sequence in other endo-β-mannanase genes from tomato (LeMAN1, LeMAN2, and LeMAN3; GenBank accession nos. AF017144, AF017438, and AF290893). Using Tag DNA polymerase (MBI Fermentas, Burlington, ON, Canada) at 2.5 mM MgCl2, the thermal cycle conditions were 94°C for 5 min followed by 35 cycles of 94°C for 45 s, 55°C for 1 min, and 72°C for 1 min 20 s. The final cycle was followed by 5 min at 72°C to complete polymerization. Twenty-eight plants of the F2 generation were then used to sequence the purified PCR fragment. An open reading frame with a high (45%–55%) identity, at the amino acid level, to the other mannanase genes from tomato was obtained. Using this sequence, a BLAST search was conducted to find an homologous expressed sequence tag clone. One (GenBank accession no. AW221434) of the 508-fwd primer region was used to design a primer at the extreme 5’ end of the gene (5’prime1, 5’-GGCTAGTCTCCTTATGATCATTATA-3’) with a NotI restriction site at its 5’ end to facilitate subsequent cloning into a vector. Using Platinum Pfx DNA polymerase (Invitrogen) and an aliquot of the aforementioned cDNA mix prepared from tomato cv Trust and tomato cv Walter mRNA, PCR was performed on both templates using 5prime1 and oligo(dT) adapter as forward and reverse primers respectively at a MgSO4 concentration of 1.5 mM (508-fwd primer). The thermal-cycle conditions were 94°C for 2 min 20 s, followed by 35 cycles of: 94°C for 30 s, 51°C for 30 s, and 68°C for 1 min 20 s. The last cycle was followed by 5 min at 68°C to complete polymerization. The PCR products were loaded onto a 1% (w/v) agarose gel; and for each, a predominant band of approximately 1,400 bp was excised, and the DNA extracted using the GFX Gel-band Purification Kit (Pharmacia, Uppsala) and digested with NotI and XhoI (XhoI site present in the oligo(dT) primer adapter sequence) and ligated into pBluescript II KS vector (Stratagene), which was cut with XhoI and XbaI. After agarose gel purification and extraction of vector and PCR fragments, ligation was performed using T4 DNA ligase (Invitrogen). Constructs were transformed into JM109 competent cells using the heat shock method (Sambrook et al., 2001), plated onto Luria-Bertani-ampicillin plates and incubated overnight at 37°C. Four of five colonies screened contained the 1,400-bp insert for both the tomato cv Trust and tomato cv Walter cDNA clones. For each clone, mini-prep plasmid DNA was sequenced using an automated sequenced (ABI, Foster City, CA) followed by interpretation using Gene Runner software (Hastings Software Inc., Hastings on Hudson, NY). The LeMAN4 protein was aligned with the three other known endo-β-mannanase amino acid sequences from tomato using the Bseasheade program (http://www.ch.embnet.org).

Genomic DNA was isolated from leaves of the following tomato cultivars: Ailsa Craig wt, Rutgers wt, Glamour, Heinz 1439, and Indian River, using the DNeasy Plant Genomic DNA kit (Qiagen USA, Valencia, CA). To amplify the gene for endo-β-mannanase from each of these cultivars, PCR was performed on 200-ng aliquots of the genomic DNA preparations using Tag DNA polymerase (MBI Fermentas) at 2 mM MgCl2. The forward primer used was 5prime2 (5’-ATATITGTAATAACCTAATCT-3’) and the reverse primer was 3’-translated region (5’-CATACTAATACCTAAAGCAG-3’), which was designed to anneal just upstream of the poly(A) tail on the cDNA clone. Thermal cycle conditions were: 94°C for 3 min 30 s, followed by 35 cycles of: 94°C for 45 s, 55°C for 40 s, and 72°C for 2 min 10 s. The last cycle was followed by 5 min at 72°C to complete polymerization. The 2,700-bp fragments were gel-purified, ligated into the pGEM T-Easy TA cloning vector (Invitrogen), and transformed into Escherichia coli. Plasmid DNA from positive clones for each cultivar was analyzed by DNA sequencing.

Genetic Cross of Tomato cv Trust and Walter

Mature anthers from tomato cv Trust flowers were rubbed upon the stigmas of emasculated tomato cv Walter flowers before anthesis, and the reverse cross was also conducted. After fruit development and ripening, seeds of tomato cv Trust-maternal, and tomato cv Walter-maternal fruits were collected, washed, and sown (eight plants of tomato cv Trust-maternal and 12 plants of tomato cv Walter-maternal). Flowers of this F1 generation were self-pollinated. Genomic DNA was extracted from the young leaves of the F1 plants using the DNeasy Genomic DNA mini-prep kit (Qiagen USA). Ripe fruit tissue was extracted and assayed for endo-β-mannanase activity using the gel-diffusion assay described earlier. Twenty-eight plants of the F2 generation were then grown from seeds of one of the tomato cv Walter-maternal plants. Young leaves and ripe fruit tissue were collected from these F2 plants and treated in the same manner as the F1 generation.

To determine the endo-β-mannanase genotypes, genomic DNA preparations were made from induced, and active F2 generations and screened by PCR using a common forward primer (LPW fwd, 5’-TTGCCAGGTTTAACTGCAAG-3’) and one of two reverse primers specific for each gene. By taking advantage of the two-nucleotide deletion in the LeMAN4 gene, the “Wr-rev” primer (5’-CTATGATACGTAGAGACCA-3’) was designed, which was only capable of extension in PCR reactions where the LeMAN4 endo-β-mannanase gene was present. The “Tr-rev” primer (5’-CTATGATAGCTTAGAGACCA-3’) conversely would only extend in PCR reactions where the LeMAN4 endo-β-mannanase gene was present. The two reverse primers contained 20 identical 5’ nucleotides, but the final 3’ nucleotides were specific to each genotype. Diagnostic PCR reactions were carried out using 200 ng of genomic DNA and Tag DNA polymerase (MBI Fermentas) at 1 mM MgCl2 with the following thermal cycle conditions: 94°C for 3 min, followed by 35 cycles of 94°C for 30 s, 62°C for 30 s, and 72°C for 30 s. The final cycle was followed by 5 min at 72°C to complete polymerization. For both sets of primers, 10 ng pBluescript+LeMAN4 (tomato cv Trust) plasmid DNA as template were included as a PCR control. Genomic DNA from tomato cv Trust and Walter plants were also included as controls. A negative control including water in place of genomic DNA was also included for both sets of primers. For each primer set, successful PCR was expected to yield a band of 351 bp, which was made visible by running the results on agarose gels containing ethidium bromide. For the F1 generation, plants 3 and 4 of the tomato cv Trust-maternal cross and plants 10 and 12 of the tomato cv Walter-maternal plants were tested in the manner described above. All 28 of the F2 generation plants were tested in the same manner.
Expression of Recombinant Proteins in E. coli and Site-Directed Mutagenesis

The coding regions (without the signal peptide) of the LeMAN4 endo-β-mannanase cDNAs (bp 109–1228) from tomato cvs Walter and Trust were amplified by PCR using a blunt-ended forward primer (pMAL-fwd, 5'-TTTCTAAAATATAATTTTTATATAAC) and a Psfl site-linked reverse primer (pMAL-rev, 5'-CGCTGACGCTATGATGCTAGAGAG). Platinum Pfx DNA Polymerase (Invitrogen) was used at 1 mm MgSO4 with the following thermal-cycle conditions: 94°C for 2 min 20 s, 35 cycles of 94°C for 25 s, 56°C for 30 s, and 68°C for 1 min 20 s. The final cycle was followed by 5 min at 68°C to complete polymerization. The products were digested with Psfl and ligated into the XmnI and Psfl sites of the MBP expression vector pMAL-c2X (New England BioLabs Inc., Beverly, MA). The empty vector and vectors containing the insert were transformed into competent E. coli cells, strain DH5α, and plated onto Luria-Bertani + ampicillin solid media. Colonies were screened by restriction analysis using purified plasmid DNA, and positives were sequenced to confirm PCR fidelity. Overnight cultures were inoculated at a concentration of 1% into rich medium + Glc and ampicillin (pMAL kit protocol, New England BioLabs Inc.) and grown at 37°C with shaking at 250 rpm. When OD600 of the culture reached an absorbance approximately 0.5, expression was induced by the addition of IPTG to 0.3 mM and further incubation for 2 h at 37°C. A crude sample of the culture was taken before the addition of IPTG (uninduced cells) and after 2 h of incubation with IPTG (induced cells). Cultures were harvested by centrifugation at 4,000 × g for 10 min; resuspended in 20 mM Tris-HCl, pH 7.4, 200 mM NaCl, and 1 mM EDTA; and frozen overnight at −20°C. Cells were thawed in ice-cold water and disrupted by sonication with a microtip probe. Sonicated cell suspensions were centrifuged at 9,000g for 20 min at 4°C, and the supernatants were retained for further analysis. Aliquots of the crude culture samples (uninduced and induced) including empty vector and vector plus inserts were separated on a 10% (w/v) polyacrylamide SDS-PAGE gel, which was stained with Coomassie Blue dye to make visible the separated proteins. A second 10% (w/v) polyacrylamide SDS-PAGE gel was run with the aforementioned samples, plus samples of the soluble proteins obtained after sonication and centrifugation. This gel was used for immuno blotting onto nitrocellulose to detect the endo-β-mannanase fusion proteins, employing the same anti-endo-β-mannanase antibody used earlier.

Site-directed mutagenesis was employed to make specific changes and/or deletions to one or more amino acids located near the carboxy terminus of the endo-β-mannanase fusion proteins. The Quik Change Site-Directed Mutagenesis System (Stratagene) was used to create the desired clones, and expression of the mutated fusion proteins was in E. coli. Equal amounts of soluble E. coli cell fractions from each clone were assayed to monitor the resulting changes in enzyme activity. The clones created are listed in Table I.

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