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cDNA Cloning of Carrot Extracellular β-Fructosidase and Its Expression in Response to Wounding and Bacterial Infection

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We isolated a full-length cDNA for apoplastic (extracellular or cell wall-bound) β-fructosidase (invertase), determined its nucleotide sequence, and used it as a probe to measure changes in mRNA as a result of wounding of carrot storage roots and infection of carrot plants with the bacterial pathogen *Erwinia carotovora*. The derived amino acid sequence of extracellular β-fructosidase shows that it is a basic protein (pl 9.9) with a signal sequence for entry into the endoplasmic reticulum and a propeptide at the N terminus that is not present in the mature protein. Amino acid sequence comparison with yeast and bacterial invertases shows that the overall homology is only about 28%, but that there are short conserved motifs, one of which is at the active site. Maturing carrot storage roots contain barely detectable levels of mRNA for extracellular β-fructosidase and these levels rise slowly but dramatically after wounding with maximal expression after 12 hours. Infection of roots and leaves of carrot plants with *E. carotovora* results in a very fast increase in the mRNA levels with maximal expression after 1 hour. These results indicate that apoplastic β-fructosidase is probably a new and hitherto unrecognized pathogenesis-related protein [Van Loon, L.C. (1985). Plant Mol. Biol. 4, 111–116]. Suspension-cultured carrot cells contain high levels of mRNA for extracellular β-fructosidase and these levels remain the same whether the cells are grown on sucrose, glucose, or fructose.

INTRODUCTION

When plants are subjected to stress as a result of wounding or invasion by pathogens, they mount a complex defense response that involves the activation of a large number of genes (reviewed by Collinge and Slusarenko, 1987). This response is being characterized at both the physiological level (increased respiration, increased ethylene production, accumulation of phytoalexins) and the molecular level (appearance of specific new gene products including phenylalanine ammonia-lyase, chitinases, β-glucanases, hydroxyproline-rich glycoproteins, protease inhibitors). This increased metabolic activity probably requires an enhanced flow of metabolizable substrates into the cells. Sucrose is the predominant form of reduced carbon transported in many plants and its metabolism is directly affected by sucrose-hydrolyzing enzymes like β-fructosidase.

Plant β-fructosidases, which cleave sucrose and related sugars into hexoses, have been studied extensively over the past decades (see, for example, Straus, 1962; Copping and Street, 1972; Ricardo, 1974; Leigh et al., 1979; Faye and Ghorbel, 1983; Krishnan et al., 1985; Laurière et al., 1988; Masuda et al., 1988; Nakamura et al., 1988; Karuppiah et al., 1989; Bracho and Whitaker, 1990; Fahrendorf and Beck, 1990; Krishnan and Pueppke, 1990). Most tissues analyzed contain multiple forms of β-fructosidase that are characterized by different subcellular locations as well as different pH optima and isoelectric points. Soluble β-fructosidases ranging in their pH optima from slightly alkaline (pH 7.5) to acidic (pH 4.5) have been described. Soluble β-fructosidases are always intracellular; they are known to be vacuolar (Leigh et al., 1979; Giaquinta et al., 1983) although it has been suggested that some are cytosolic (Karuppiah et al., 1989; Fahrendorf and Beck, 1990). Insoluble β-fructosidase, with a pH optimum between pH 4.0 and pH 5.3, is ionically bound to the cell wall and can be solubilized by extracting cell walls with high salt (Strauss, 1962; Fahrendorf and Beck, 1990). In carrots, the soluble and insoluble (cell wall) β-fructosidases show weak immunogenic cross-reactivity (Laurière et al., 1988), indicating a distant relationship between the respective proteins.

High activity of soluble β-fructosidase is often found in young seedlings (Cooper and Greenshields, 1964; Ricardo and ap Rees, 1970), in tuberous roots, which do not store

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much sucrose (Ricardo and Sovia, 1974), and in mature fruits (Arnold, 1965; Nakagawa et al., 1971; Krishnan and Pueppke, 1990). It has been proposed that the soluble β-fructosidases participate in the regulation of the hexose level in mature tissues (Ricardo, 1974; Ricardo and Sovia, 1974) and in the utilization of sucrose stored in vacuoles (Leigh et al., 1979).

High extracellular β-fructosidase activity is usually found in rapidly growing tissue with a high demand for hexoses such as extension zones of root tips and extending internodes, at sites of emerging secondary roots, in developing tap roots, and in developing leaves (Eschrich, 1980). When cell growth ceases, the activity of the extracellular β-fructosidase decreases and finally disappears. It has been suggested that the extracellular β-fructosidase plays a role in phloem unloading by maintaining a steep sucrose concentration gradient between source (photosynthetic leaf tissue) and sink (heterotrophic cells) regions of a plant (Eschrich, 1980; Morris and Arthur, 1985).

Sucrose is taken up either after hydrolysis into glucose and fructose or as sucrose. Sucrose may enter cells by different mechanisms: sink cells from pea roots (Dick and ap Rees, 1975), from corn roots (Giaquinta et al., 1983), and from developing leaves from sugar beet (Schmalstig and Geiger, 1985) and from tobacco (Turgeon, 1987) take up sucrose by symplastic transport via plasmodesmata. Sucrose enters sink cells from sugar beet tap roots (Buckhout, 1989) and from soybean cotyledons (Ripp et al., 1988) by way of a sucrose/proton antiporter. Surprisingly, in the case of sucrose uptake without preceding hydrolysis, extracellular β-fructosidase activity was detected (see, for example, Giaquinta et al., 1983; Lemoine et al., 1988). However, because there are fundamental differences in the cellular photoassimilate uptake mechanisms in different plant species and different plant organs, there is no generally accepted function of extracellular β-fructosidase.

In addition to the regulation of the β-fructosidase activity during plant development, the enzyme activity is also affected by environmental stimuli. Both high soluble and high insoluble β-fructosidase activities were induced by wounding of storage roots from carrots, sweet potato, and red beets (Bacon et al., 1965; Edelman and Hall, 1965; Ricardo and ap Rees, 1970; Matsushita and Uritani, 1974; Leigh et al., 1979) and by pathogen infection (Billett et al., 1977; Callow et al., 1980; Krishnan and Pueppke, 1988). The role of β-fructosidases in wound response and in pathogen response is unclear.

In contrast to the rapidly growing body of information on different plant β-fructosidases (see above), there is no information on their protein structures, on their genes, and on their regulation during plant development or by environmental stimuli. We present here the primary amino acid sequence of carrot cell wall β-fructosidase deduced from a full-length cDNA clone, its comparison with β-fructosidases from bacteria and yeast, and the regulation of β-fructosidase gene expression by wounding and by pathogen infection.

RESULTS

Isolation of a Full-Length cDNA Clone from a λgt11 Expression Library

Cell wall β-fructosidase was purified by conventional chromatography techniques from a carrot cell suspension culture [wild carrot cell culture line W001C, *Daucus carota*, Queen Anne's lace (Sung, 1976)] as described (Laurière et al., 1988). The identity of the purified protein was verified by an assay for β-fructosidase activity. The purified polypeptide migrated on a denaturing polyacrylamide gel as a single band with a relative molecular weight of 63,000. The N-terminal amino acid sequence was determined by automated Edman degradation (Edman and Begg, 1967); the amino acid sequence obtained is underlined in Figure 1.

Carrot cell wall β-fructosidase is an N-linked glycoprotein with high-mannose and complex-type oligosaccharides. Three tryptic glycopeptides were isolated from purified ^{3}H-glucosamine-labeled cell wall β-fructosidase by reverse-phase HPLC as described (Laurière et al., 1988). Partial amino acid sequences were determined by automated Edman degradation. The sequences obtained are underlined in Figure 1.

Polyclonal antibodies were raised against the deglycosylated polypeptide. Affinity-purified anti-β-fructosidase IgG proved to be highly specific when tested by immunoblot analysis (Laurière et al., 1989).

The level of β-fructosidase mRNA throughout a carrot cell culture life cycle was measured at 1-day intervals by in vitro translation of total RNA followed by immunoprecipitation, SDS-PAGE, and fluorography. Translatable β-fructosidase mRNA was present at all timepoints but reached a maximum between day 3 and day 4 after cell culture subcultivation (data not shown). The maximum level of β-fructosidase mRNA correlated with the onset of rapid cell growth in culture.

cDNA was synthesized from poly(A^{+}) RNA extracted from a 3.5-day-old cell culture and was size fractionated by agarose gel electrophoresis. cDNA with a length of 1.1 kb to 2.3 kb was used to construct a λgt11 expression library. When this library was screened with the affinity-purified anti-β-fructosidase IgG (Young and Davis, 1983), we obtained one positive clone per 20,000 plaque-forming units. Twelve antibody-positive λ clones were rescreened with a synthetic oligonucleotide probe derived from one of the glycopeptide sequences (Albertsen et al., 1988). One positive clone was obtained. Its cDNA insert was used to rescreen the λgt11 library for a full-length clone. Both strands of a full-length cDNA clone were sequenced by

the dideoxynucleotide chain-termination method (Messing, 1983).

Nucleotide Sequence of the cDNA Clone and Analysis of the cDNA-Derived Amino Acid Sequence

The complete nucleotide sequence of the full-length cDNA (2.0 kb) is shown in Figure 1. It contains one open reading frame starting at nucleotide 34 with an ATG start codon and ending at nucleotide 1809 before a TAA stop codon. The open reading frame encodes a polypeptide chain of 592 residues with a calculated molecular weight of 66,813. All four partial amino acid sequences obtained from the purified protein (the N terminus and the three tryptic peptide sequences) are present in the cDNA-derived amino acid sequence. This confirms that the cDNA clone encodes carrot cell wall β-fructosidase.

In addition to the open reading frame, the cDNA also contains 33 bp of 5′-untranslated and 183 bp of 3′-untranslated sequence with a poly(A) tail. A putative translation initiation signal (ATG) of the β-fructosidase cDNA is located at nucleotide positions 34 to 36 (Joshi, 1987; Lütcke et al., 1987). It is the only methionine codon between a stop codon (TAA) close to the 5′ end of the cDNA and the DNA sequence encoding the N terminus of the mature β-fructosidase polypeptide (nucleotide 178), which indicates that this ATG is the translation initiation signal used.

The consensus signal for polyadenylation AATAAA, which is usually located 10 nucleotides to 30 nucleotides upstream from the poly(A) tail, is lacking in the β-fructosidase cDNA. It might be replaced by the sequence AATAAT [nucleotides 1944 to 1949 of the cDNA sequence located 23 nucleotides upstream of the poly(A) tail; see Figure 1]. Such a deviation from the consensus sequence theme was also shown for several other plant mRNAs (Heidecker and Messing, 1986).

Carrot cell wall β-fructosidase is an N-linked glycoprotein with one high-mannose type and two complex-type glycans per polypeptide (A. Sturm, unpublished results).

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TTCCTTCAAATCTAATTCTCTGAAATTCCAGGGATGGGTGTAACAATAAGAAACCGTAAC     60
                                   MetGlyValThrIleArgAsnArgAsn

TATGATCACGGCTCTCTCCCGTTTCTTCAATCTCTCCTAGCCATCTTGCTCGTAACAACC    120
TyrAspHisGlySerLeuProPheLeuGlnSerLeuLeuAlaIleLeuLeuValThrThr

ACCACATTACACATTAACGGCGTCGAAGCCTTCCACGAAATCCACTATAATCTCCAGTCT    180
ThrThrLeuHisIleAsnGlyValGluAlaPheHisGluIleHisTyrAsnLeuGlnSer
                                                          ↑
GTTGGTGCTGAGAATGTAAAGCAAGTCCACAGAACTGGCTACCACTTTCAACCTAAACAA    240
ValGlyAlaGluAsnValLysGlnValHisArgThrGlyTyrHisPheGlnProLysGln

AACTGGATTAACGATCCAAATGGACCAATGTATTATAAGGGAGTATATCATCTTTTTTAT    300
AsnTrpIleAsnAspProAsnGlyProMetTyrTyrLysGlyValTyrHisLeuPheTyr

CAGTACAATCCCAAGGGTGCAGTGTGGGGTAATATTGTATGGGCCCATTCGGTGTCGACT    360
GlnTyrAsnProLysGlyAlaValTrpGlyAsnIleValTrpAlaHisSerValSerThr

GACTTGATCAACTGGACACCGCTCGAGCCTGCAATCTTTCCATCCAAACCATTTGATAAA    420
AspLeuIleAsnTrpThrProLeuGluProAlaIlePheProSerLysProPheAspLys
        *********
TATGGATGCCGGTCCGGTTCAGCGACTATCCTTCCAGGTAATAAGCCTGTAATACTCTAT    480
TyrGlyCysArgSerGlySerAlaThrIleLeuProGlyAsnLysProValIleLeuTyr

ACTGGAATTGTAGAAGGCCCTCCAAAAAATGTACAAGTTCAAAACTATGCTATTCCCGCC    540
ThrGlyIleValGluGlyProProLysAsnValGlnValGlnAsnTyrAlaIleProAla

AACCTTTCTGATCCTTACCTTCGGAAGTGGATCAAACCCGATAACAACCCCTTAGTCGTT    600
AsnLeuSerAspProTyrLeuArgLysTrpIleLysProAspAsnAsnProLeuValVal
*********
GCCAACAATGGGGAAAATGCCACAGCTTTCCGGGACCCGACCACAGCATGGTTGGACAAA    660
AlaAsnAsnGlyGluAsnAlaThrAlaPheArgAspProThrThrAlaTrpLeuAspLys
              *********
AGTGGGCACTGGAAAATGTTGGTTGGTAGTAAGAGGAACCGAAGAGGAATTGCGTATTTG    720
SerGlyHisTrpLysMetLeuValGlySerLysArgAsnArgArgGlyIleAlaTyrLeu

TACAGGAGTAAAGATTTTATTAAATGGACCAAGGCTAAACACCCGATCCATTCCCAGGCT    780
TyrArgSerLysAspPheIleLysTrpThrLysAlaLysHisProIleHisSerGlnAla

AACACTGGAATGTGGGAATGTCCTGATTTTTTCCCAGTATCACTGAAGGGATTGAACGGA    840
AsnThrGlyMetTrpGluCysProAspPhePheProValSerLeuLysGlyLeuAsnGly

TTAGATACTTCTGTAACAGGAGAAAGTGTTAAGCATGTGCTGAAGGTTAGCTTAGATTTA    900
LeuAspThrSerValThrGlyGluSerValLysHisValLeuLysValSerLeuAspLeu

ACAAGGTACGAATATTACACCGTTGGTACATATCTTACTGATAAGGACAGGTATATCCCG    960
ThrArgTyrGluTyrTyrThrValGlyThrTyrLeuThrAspLysAspArgTyrIlePro

GACAATACATCAGTTGATGGTTGGGCCGGTTTGAGGTATGATTATGGAAACTTTTACGCA   1020
AspAsnThrSerValAspGlyTrpAlaGlyLeuArgTyrAspTyrGlyAsnPheTyrAla
   *********
TCAAAGACGTTCTTTGATCCTTCCAAGAATCGGAGAATTCTGTGGGGTTGGGCTAATGAA   1080
SerLysThrPhePheAspProSerLysAsnArgArgIleLeuTrpGlyTrpAlaAsnGlu
                                                      ******
TCTGACAGTACTGCTCATGATGTTGCAAAGGGATGGGCTGGAATTCAGTTAATTCCACGG   1140
SerAspSerThrAlaHisAspValAlaLysGlyTrpAlaGlyIleGlnLeuIleProArg
***
ACACTTTGGTTGGATCCAAGCGGGAAACAATTGATGCAGTGGCCAATCGAAGAATTAGAA   1200
ThrLeuTrpLeuAspProSerGlyLysGlnLeuMetGlnTrpProIleGluGluLeuGlu

ACACTAAGAGGGTCAAAAGTTAAATTTTCCAGAAAACAAGATCTGAGTAAGGGAATTCTT   1260
ThrLeuArgGlySerLysValLysPheSerArgLysGlnAspLeuSerLysGlyIleLeu

GTTGAAGTAAAAGGAATTACAGCTGCACAGGCTGATGTCGAGGTGACATTTTCATTTAAA   1320
ValGluValLysGlyIleThrAlaAlaGlnAlaAspValGluValThrPheSerPheLys

AGTTTGGCGAAACGTGAGCCATTTGATCCGAAATGGCTAGAATATGATGCTGAAAAGATT   1380
SerLeuAlaLysArgGluProPheAspProLysTrpLeuGluTyrAspAlaGluLysIle

TGCAGTCTCAAGGGCTCTACAGTTCAAGGTGGAGTTGGACCATTTGGCCTCTTAACACTG   1440
CysSerLeuLysGlySerThrValGlnGlyGlyValGlyProPheGlyLeuLeuThrLeu

GCTTCAGAAAAGCTAGAAGAATACACTCCTGTCTTCTTCAGAGTGTTCAAAGCACAAAAC   1500
AlaSerGluLysLeuGluGluTyrThrProValPhePheArgValPheLysAlaGlnAsn

ACGCATAAAGTTCTTATGTGCTCTGATGCAACCCGGTCCTCGCTAAAGGAAGGATTGTAC   1560
ThrHisLysValLeuMetCysSerAspAlaThrArgSerSerLeuLysGluGlyLeuTyr

AGACCGTCATTTGCAGGTTTTGTCGATGTTGACTTGGCCACTGATAAGAAAATCTCTCTT   1620
ArgProSerPheAlaGlyPheValAspValAspLeuAlaThrAspLysLysIleSerLeu

AGAAGCTTGATTGATAATTCGGTTGTCGAGAGTTTTGGAGCTAAAGGAAAGACATGCATA   1680
ArgSerLeuIleAspAsnSerValValGluSerPheGlyAlaLysGlyLysThrCysIle

TCATCTAGGGTGTATCCAACACTAGCTGTTTATGAGAATGCTCATTTATATGTCTTCAAC   1740
SerSerArgValTyrProThrLeuAlaValTyrGluAsnAlaHisLeuTyrValPheAsn

AATGGCTCAGAGACTATTACCGTAGAGAATCTGGATGCATGGAGCATGAAGAAGCCCTTG   1800
AsnGlySerGluThrIleThrValGluAsnLeuAspAlaTrpSerMetLysLysProLeu
*********
CGAATGAACTAACCAATGCAGGAAGAAAACGTACATGGAAAATATTTTGACTTCTGTAAT   1860
ArgMetAsn

TTTGTTTTTCATCCCGGAATAGTCATGCCCACATTTGAATACAATGCTAGAGCTGTCTGT   1920

CACATGATCAATTCACTTTATATAATAATATATTTCTACCTATTTAGGTTTTAAAAAAAA   1980
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AAAAAAAAAAAGGAA  1995
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Figure 1. Nucleotide Sequence of the Carrot Cell Wall β-Fructosidase cDNA.

Nucleotides are numbered from the first base of the cDNA clone. The dashed line (– – –) denotes a potential polyadenylation signal. The deduced amino acid sequence for carrot cell wall β-fructosidase is indicated below the nucleotide sequence. The underlined sequences denote the amino acid sequences determined by automated Edman degradation (N-terminal sequence of the mature polypeptide and three partial tryptic glycopeptide sequences, respectively). The arrow (↑) shows the first amino acid residue of the mature polypeptide. The sequences marked by asterisks (*) denote potential N-glycosylation sites (site 1 to 6, respectively). Complex-type glycans are linked to glycosylation sites 2 and 4; a high-mannose-type glycan is attached to glycosylation site 5.

Thus, only three out of the six potential glycosylation sites (Asn-X-Ser/Thr) present in the amino acid sequence are actually used. The six potential glycosylation sites are marked in Figure 1 by asterisks. The three glycans contribute about 5000 D to the electrophoretic mobility (Laurière et al., 1988). Hence, the deglycosylated β-fructosidase with a relative molecular weight of 58,000 is about 9000 D smaller than the cDNA-encoded polypeptide. This discrepancy can be explained in part by the removal of a 48-amino-acid-long peptide equivalent to about 5000 D from the N terminus (the mature polypeptide starts with amino acid residue 49 marked by an arrow in Figure 1) during a polypeptide maturation process. In addition, cell wall β-fructosidase is a highly charged polypeptide containing 57 negative and 85 positive amino acid residues (the calculated isoelectric point is 9.88) and may run somewhat anomalously on SDS-PAGE.

Cell wall β-fructosidase has a long signal peptide with a basic N-terminal region (n-region) followed by 20 hydrophobic amino acid residues (h-region) and a polar C-terminal region (c-region), as detailed in Figure 2. The N terminus of the mature polypeptide, which starts with amino residue 49 of the cDNA-derived amino acid sequence, is not adjacent to a typical signal peptide cleavage site (Von Heijne, 1983, 1986). By applying the Von Heijne rules, the predicted signal peptide cleavage site would be located most likely between amino acid residues 31 and 32 or between residues 39 and 40 (see arrows in Figure 2).

Comparison of Carrot Cell Wall β-Fructosidase with β-Fructosidases from *Saccharomyces cerevisiae* and from *Bacillus subtilis*

The amino acid sequence of carrot cell wall β-fructosidase was used to search the protein sequence data banks (National Biomedical Research Foundation PIR Protein Library and PIR New Library and the European Molecular and Biological Laboratory Swissprot Library). Homologies were found to two β-fructosidases from *B. subtilis* (levanase and sucrase) and to invertase from the yeast *S. cerevisiae*. A comparison of these sequences by dot matrix analysis showed that the homologies are restricted to the N-terminal halves of the polypeptides. The different β-fructosidases share only a few homologies in their C-terminal domains (data not shown; the homologies between levanase, sucrase, and invertase are discussed in detail by Martin et al., 1987).

The carrot cell wall β-fructosidase amino acid sequence up to amino acid residue 400 was compared directly with those from levanase, sucrase, and invertase, as shown in Figure 3. Gaps were introduced for optimal sequence alignment. The mature polypeptides of the secreted β-fructosidases (carrot β-fructosidase, levanase, and yeast invertase; sucrase is a cytoplasmic protein) start 20 amino acids in front of a highly conserved peptide domain common to all β-fructosidases (β-fructosidase motif boxed in Figure 3). Within the first 100 amino acids of the mature β-fructosidases, highest homology was found between carrot β-fructosidase and levanase from *B. subtilis* (44% identity). Thirty-seven percent of the amino acids were identical when the N terminus of carrot cell wall β-fructosidase was compared with sucrase or with yeast invertase. Despite the homology between carrot β-fructosidase and levanase, the carrot enzyme failed to hydrolyze levan from *Aerobacter levaniucum* [β(2→6)polyfructan] or inulin from chicory root [β(2→1)polyfructan] (data not shown), whereas levanase completely hydrolyses both types of polysaccharides (Martin et al., 1987). In contrast, all four β-fructosidases compared hydrolyze sucrose with high efficiency. In addition, the carrot cell wall enzyme hydrolyzes raffinose with 70% efficiency when compared with sucrose as a substrate.

The sucrose hydrolyzing activity of carrot cell wall β-fructosidase was inhibited by *p*-hydroxymercurybenzoate (data not shown), which may indicate that a cysteine residue is important for the catalytic activity. This is in agreement with the finding of Martin et al. (1987), who showed that cysteine residue 224 is an essential part of the catalytic domain of levanase. This cysteine residue and its neighboring amino acids are conserved in all four β-fructosidases (see Cys residue 256 of cell wall β-fructosidase, marked by an arrow in Figure 3).

Both carrot cell wall β-fructosidase (Laurière et al., 1988) and yeast invertase (Reddy et al., 1988) are glycoproteins with N-linked oligosaccharides. A comparison of the positions of the glycosylation sites (six in the case of carrot β-fructosidase and 14 in the case of yeast invertase; potential glycosylation sites located in the N-terminal half

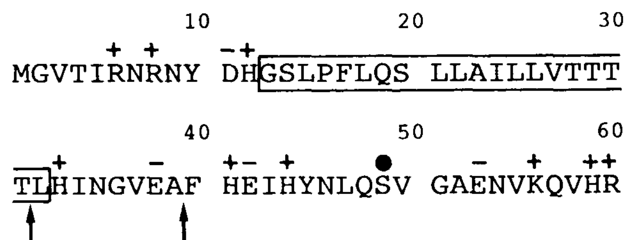

Figure 2. The First 60 Residues of the Amino Acid Sequence Deduced from the Nucleotide Sequence of the Carrot Cell Wall β-Fructosidase cDNA.

The amino acids are in one-letter code. Basic amino acids are indicated by a plus symbol (+) and acidic amino acids are indicated by a minus symbol (−). A hydrophobic peptide domain is boxed. Potential signal peptide cleavage sites are indicated by arrows (↑). The first amino acid residue of the mature β-fructosidase polypeptide is marked by a dot (●).

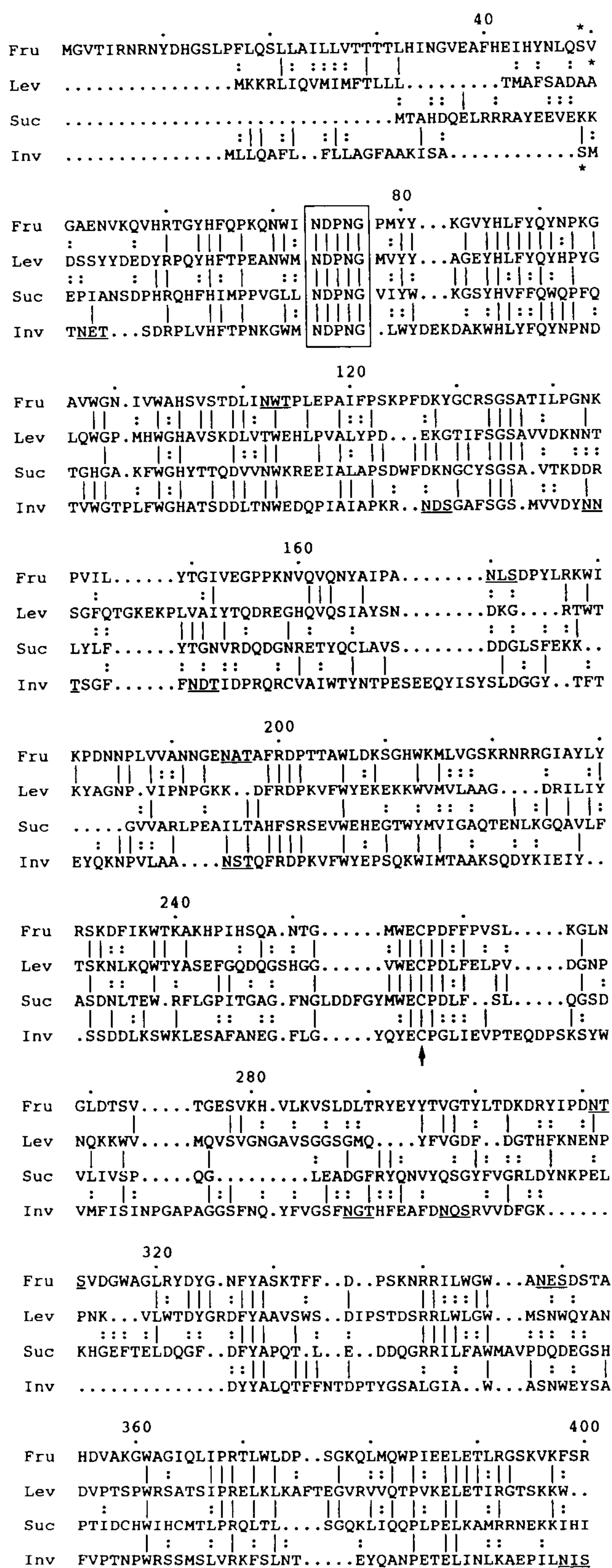

Figure 3. Comparison of the Amino Acid Sequence from Carrot Cell Wall β-Fructosidase (Fru) with the Amino Acid Sequences from Levanase (Lev) and Sucrase (Suc) from *B. subtilis* and Invertase (Inv) from Yeast.

Only the first 400 amino acids from the carrot β-fructosidase sequence are shown. The amino acid sequences of the four proteins are in one-letter code and have been aligned by introducing gaps (. . .) to maximize identities. Vertical bars indicate identical residues and colons indicate conservative replacements. The first amino acid residue of the mature secreted polypeptides (Fru, Lev, and Inv) is marked by asterisks (*). The potential glycosylation sites from carrot cell wall β-fructosidase and from yeast invertase are underlined. The boxed region represents a peptide domain that is conserved in all four β-fructosidases (β-fructosidase motif). The arrow (↑) marks a cysteine residue that is conserved in all four β-fructosidases and that seems to be important for the catalytic activity.

of the two proteins are underlined in Figure 3) shows that only one position is conserved. However, in the carrot protein, this conserved glycosylation site is not used.

Genomic DNA Gel Blot

To determine the number of cell wall β-fructosidase genes in the diploid wild carrot genome, DNA gel blot analyses (Southern, 1975) were performed. The labeled 5′ half (EcoRI/EcoRI, 1100 bp) or the 3′ half (EcoRI/EcoRI, 800 bp) of the cDNA hybridized to a few restriction fragments of genomic DNA, revealing the presence of one or at the most two genes, as seen in Figures 4A and 4B, respectively. This finding was confirmed by a gene reconstruction experiment with EcoRI-restricted carrot DNA and purified β-fructosidase cDNA titered at 0.5-copy, 1.0-copy, 2.0-copy, 3.0-copy, and 5.0-copy equivalents per haploid carrot genome (K. Ramloch and A. Sturm, unpublished results). At the washing stringency used (0.1 × SSC and 0.5% SDS, 1 hr at 65°C), most likely we do not pick up the gene(s) for vacuolar β-fructosidase.

Induction of β-Fructosidase mRNA Accumulation in Carrots by Wounding

Wounding of storage tissues from carrots, sweet potato, and red beets has been shown to result in a slow rise in the activity of β-fructosidase (Ricardo and ap Rees, 1970; Matsushita and Uritani, 1974; Leigh et al., 1979). To determine whether wounding increases the levels of mRNA for β-fructosidase, we incubated slices of phloem parenchyma tissue of maturing carrots in sterile water with vigorous shaking, isolated total RNA at specific times after tissue wounding, as shown in Figure 5, and analyzed mRNA levels on RNA gel blots. Equal loading of RNA per lane was confirmed by the equal intensities of the ribosomal RNAs on the ethidium bromide-stained agarose gel (data not shown).

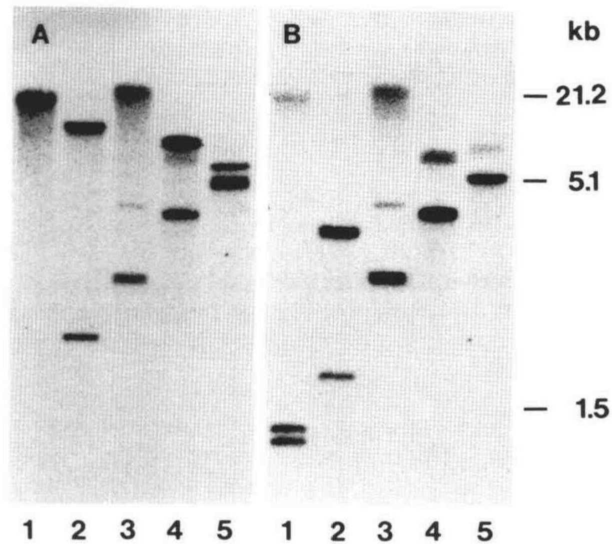

Figure 4. DNA Gel Blot Analysis of Cell Wall β-Fructosidase Sequences in the Carrot Genome.

(A) Carrot genomic DNA (10 μg/lane) was digested with BamHI (lane 1), EcoRI (lane 2), KpnI (lane 3), SphI (lane 4), and XbaI (lane 5). The fragments were separated by agarose gel electrophoresis and blotted before hybridization with the ^{32}P-labeled 5′ half of the carrot cell wall β-fructosidase cDNA.

(B) The same blot was probed with the ^{32}P-labeled 3′ half of the carrot cell wall β-fructosidase cDNA.

The level of β-fructosidase mRNA was determined by probing the RNA gel blot with the carrot β-fructosidase cDNA. As shown in Figure 5A, maturing, unwounded carrots have no detectable cell wall β-fructosidase mRNA. After a lag phase of about 2 hr, β-fructosidase mRNA started to accumulate. β-Fructosidase mRNA was maximally induced 12 hr after wounding, followed by a slow decline over the next 60 hr. (Figure 5 shows the results only up to 24 hr after wounding.)

We also measured the accumulation of β-fructosidase activity in the same tissue. The tissue slice homogenates were separated into soluble proteins and cell wall-bound proteins and the β-fructosidase activity was measured. The data in Figure 6 show an increase in both cell wall-bound β-fructosidase activity and soluble β-fructosidase activity starting about 12 hr after wounding. Both enzymes have acidic pH optima and were measured at pH 4.5. Within 40 hr, both β-fructosidases increased about fivefold above control levels before their activities declined again. Both enzymes increased to about the same level when expressed as units of enzyme activity per gram, fresh weight. The accumulation of cell wall β-fructosidase mRNA in wounded carrots was compared with the accumulation of mRNA for the hydroxyproline-rich glycoprotein (HRGP) and for phenylalanine ammonia-lyase (PAL), two well-characterized wound-inducible and pathogen-inducible poly-

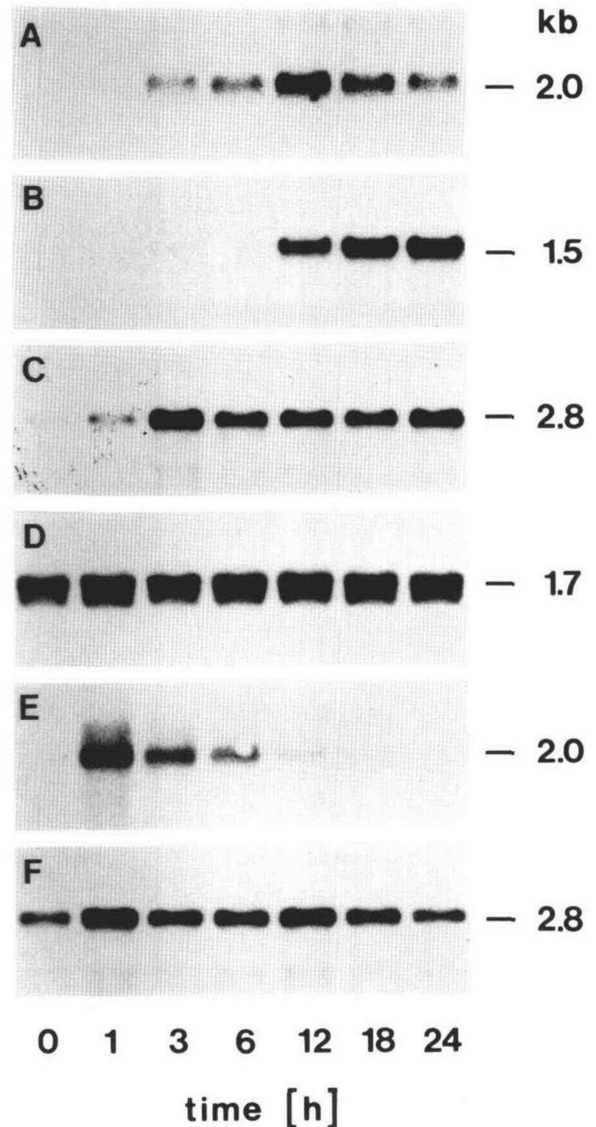

Figure 5. Time Course of RNA Accumulation in Carrot Roots in Response to Wounding [**(A)** to **(C)**] and to Infection [**(E)** and **(F)**].

The timepoints refer to time after treatment when RNA was isolated.

(A) Maturing carrot roots were mechanically wounded. Total RNA (10 μg/lane) was separated on formaldehyde containing agarose gels and was transferred to a nylon membrane. The blot was probed with carrot cell wall β-fructosidase cDNA.

(B) An identical blot was probed with a genomic carrot HRGP clone.

(C) An identical blot was probed with parsley PAL cDNA.

(D) Maturing carrot roots were infected by spraying with a suspension of *E. carotovora* in water. Poly(A^+) RNA (5 μg/lane) was separated on a formaldehyde-containing agarose gel and was transferred to a nylon membrane. The blot was probed with a genomic soybean actin clone.

(E) An identical blot was probed with carrot cell wall β-fructosidase cDNA.

(F) An identical blot was probed with parsley PAL cDNA.

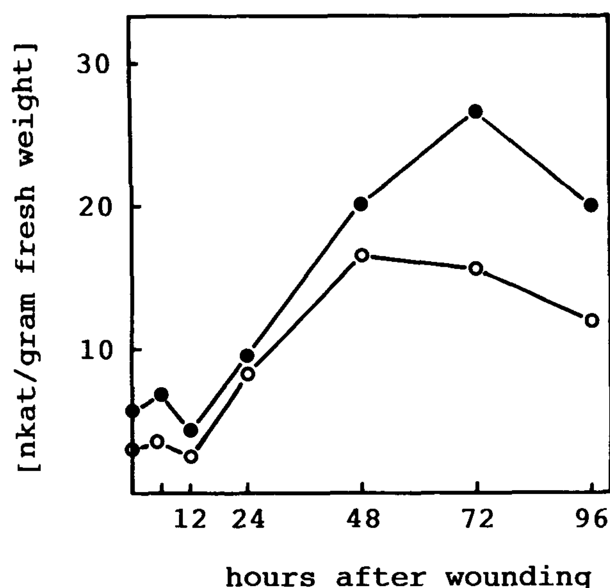

Figure 6. Timecourse of the Induction of β-Fructosidase Activity by Wounding.

Discs of carrot phloem parenchyma tissue were incubated in water on a rotary shaker for 1 hr to 96 hr. The carrot disc proteins were separated in soluble (○) and cell wall-bound (●) proteins, and the β-fructosidase activity was determined at pH 4.5. The enzyme activity is expressed in nanokatals per gram, fresh weight.

peptides (see, for example, Corbin et al., 1987; Lois et al., 1989). RNA gel blots with total RNA from aerated tissue slices were probed with a genomic clone coding for HRGP from carrot and with a cDNA encoding PAL from parsley (Figures 5B and 5C, respectively). The amounts of HRGP and PAL mRNA in unwounded maturing carrots were low or below the detection limit. HRGP mRNA started to accumulate after a lag phase of more than 3 hr, followed by a steady increase throughout the experiment (Figure 5B). In contrast to the slow HRGP accumulation, the accumulation of PAL mRNA was very rapid and reached a maximum 3 hr after wounding, followed by a slow decline over the next 20 hr (Figure 5C).

The effect of wounding on the expression of a housekeeping gene was analyzed by probing the RNA gel blot with a genomic fragment encoding soybean actin. A hybridization signal (doublet that migrated on the RNA gel in front of the ribosomal small subunit rRNA) with equal intensity in all 10 lanes was obtained, indicating that actin gene expression was not altered by wounding (data not shown).

Induction of β-Fructosidase Gene Expression in Carrots by Infection with *Erwinia carotovora*

Whole carrot plants were sprayed with a suspension of *E. carotovora* in water. Total RNA was isolated at specific times after infection (see Figure 5) and was used for the preparation of poly(A^+) RNA.

Five micrograms of root poly(A^+) RNA per timepoint (as determined by the optical density at 260 nm) were analyzed on an RNA gel blot using a genomic fragment encoding soybean actin as a probe (Figure 5D). All seven lanes show a doublet with equal intensities that migrated in front of the small rRNA. Thus, the expression of the carrot actin genes was not affected by the bacterial pathogen.

The RNA gel blot was subsequently probed with the cDNA encoding the carrot β-fructosidase and with a cDNA encoding PAL from parsley (Figures 5E and 5F, respectively). Roots from control plants (timepoint 0) had no detectable mRNA encoding β-fructosidase but had some PAL mRNA. One hour after spraying with the pathogen, there was massive accumulation of β-fructosidase mRNA, followed by a decline back to control plant levels within 20 hr (Figure 5E). The accumulation of PAL mRNA was also very fast and reached a first maximum 1 hr after spraying. A second but less pronounced maximum of PAL mRNA accumulation was observed 12 hr after spraying, followed by a decrease back to control levels after an additional 12 hr (Figure 5F). [A quantitative comparison of the hybridization signals from the wounding experiment with the hybridization signals from the infection experiment is difficult because in the first case total RNA was used (Figures 5A to 5C) and only poly(A^+) RNA in the second (Figures 5D to 5F)].

An RNA gel blot with poly(A^+) RNA from infected leaves was probed with the cDNA encoding carrot β-fructosidase. The result showed that the accumulation of β-fructosidase mRNA in leaves was qualitatively and quantitatively identical to the β-fructosidase mRNA accumulation in roots (data not shown).

Throughout the experiment, the pathogen-sprayed plants did not show severe signs of infection, although the roots changed slightly in color from orange to orange-brown. To confirm that the *Erwinia* strain used was pathogenic, carrot roots were scratched with a scalpel before spraying with the *Erwinia* suspension. These wounded carrots disintegrated within 24 hr to 36 hr at 27°C to a brown mash.

Five micrograms of *E. carotovora* RNA were probed with the carrot β-fructosidase cDNA. No hybridization signal was obtained. Carrots sprayed only with water showed no elevated levels of β-fructosidase mRNA (data not shown).

Levels of β-Fructosidase mRNA in Carrot Cell Suspension Cultures Grown on Sucrose, Glucose, or Fructose

To test whether the expression of cell wall β-fructosidase is regulated by its substrate (sucrose) or by the hydrolysis products (glucose and fructose), the carrot cell suspension culture W001C was maintained over several months on

either sucrose, glucose, or fructose by transferring the cells at 1-week intervals into fresh Murashige and Skoog medium (MS medium). The cells grown on different carbohydrates showed no obvious changes in their growth rate or their morphology. Total RNA was isolated from cells 3 days after subculturing. The β-fructosidase mRNA levels were determined by RNA gel blot analysis using the carrot β-fructosidase cDNA as a probe. Figure 7 shows that carrot cells grown on glucose as the sole carbon source have a slightly higher β-fructosidase mRNA content (lane 2) than cells grown on fructose or on sucrose (lanes 1 and 3, respectively).

DISCUSSION

Identification of the cDNA for Extracellular β-Fructosidase

Carrots contain an extracellular form and a soluble form of β-fructosidase that differ by their relative molecular weights and their pH optima of enzyme activity. The extracellular carrot β-fructosidase is ionically bound to the cell wall (Laurière et al., 1988). The weak immunological cross-reactivity between the extracellular and the soluble carrot β-fructosidase indicates a distant relationship between the two polypeptides. Thus, the different carrot β-fructosidases are most likely encoded by different but related genes.

We isolated a full-length cDNA clone encoding extracellular carrot β-fructosidase. The four partial peptide sequences obtained from the purified β-fructosidase polypeptide (the N terminus and three tryptic glycopeptide sequences) allowed us to confirm the identity of the cDNA clone. Genomic DNA gel blots and a gene reconstruction experiment showed that carrot cell wall β-fructosidase is encoded by one or at the most by two genes. Under the hybridization and washing conditions used, we do not expect to pick up the gene(s) coding for the soluble enzyme.

β-Fructosidase is a highly charged polypeptide with a calculated isoelectric point of 9.88. The net positive charge might be responsible for the ionic interaction of β-fructosidase with a negatively charged cell wall component such as rhamnogalacturonan (Albersheim, 1975).

Sites for Cotranslational and Post-translational Modification

The cDNA-derived amino acid sequence for carrot cell wall β-fructosidase has six potential glycosylation sites and three of these sites are used. Cell wall β-fructosidase is an N-linked glycoprotein with one high-mannose and two complex-type glycans per polypeptide. The presence of high-mannose and complex-type glycans indicates that β-fructosidase is synthesized on the rough endoplasmic reticulum where N-glycosylation takes place (Lehle and Tanner, 1983) and that it is transported into the apoplast by way of the Golgi apparatus where N-glycan modification occurs (Chrispeels, 1983; Sturm et al., 1987). The proposed biosynthetic pathway is supported by the presence of a signal peptide-like sequence (Von Heijne, 1986) at the N terminus of the cDNA-derived amino acid sequence. Two predicted signal peptide cleavage sites are located downstream of the hydrophobic peptide domain (h-region), whereas the N terminus of the mature polypeptide starts even further downstream. Although we cannot rule out the loss of a small N-terminal fragment during protein purification, this finding suggests that carrot cell wall β-fructosidase might be synthesized as a preproprotein. The function of the putative β-fructosidase propeptide is not clear, but in analogy to other preproenzymes it might be involved in the regulation of enzyme activity (Haselik and Tanner,

Figure 7. RNA Gel Blot Analysis of Total RNA from Carrot Suspension Cells Grown for Several Months on Fructose (Fru), Glucose (Glu), or Sucrose (Suc).

RNA (10 μg/lane) was separated on formaldehyde-containing agarose gels and was transferred to a nylon membrane. The blot was probed with the carrot cell wall β-fructosidase cDNA.

1978). The proposed processing pathway for carrot cell wall β-fructosidase resembles the proteolytic processing of the tomato cell wall protein polygalacturonase, from which a 24-amino-acid-long signal peptide is removed cotranslationally and a highly charged 47-amino-acid-long prosequence is removed post-translationally (DellaPenna and Bennett, 1988).

Sequence Identities with Other β-Fructosidases

Carrot cell wall β-fructosidase shows significant sequence homology with β-fructosidases from yeast and from bacteria in the N-terminal portion of the proteins. Martin et al. (1987) speculated that the different β-fructosidases have evolved by fusion of two DNA segments corresponding to the N-terminal and C-terminal parts of the proteins and that the N-terminal part of these proteins harboring the catalytic activity derived from a common ancestor. The C-terminal part of the extracellular carrot enzyme may participate in the interaction of the polypeptide with cell wall components.

The greatest sequence homology was found between the amino acid sequences deduced for carrot cell wall β-fructosidase and levanase from *B. subtilis*. The functional relationship between carrot cell wall β-fructosidase and levanase seemed to be restricted to their ability to hydrolyze sucrose because the carrot enzyme failed to hydrolyze the polyfructans levan and inulin, whereas levanase did. Both carrot cell wall β-fructosidase and levanase are inhibited by *p*-hydroxymercurybenzoate, indicating the presence of a cysteine residue in the catalytic center. This cysteine residue and the surrounding amino acids are conserved in the primary sequences of all known β-fructosidases.

Lack of Substrate Regulation

In the carrot cell suspension culture, the expression of the cell wall β-fructosidase gene seems to be independent of the nature of the carbohydrate source. Equally high levels of cell wall β-fructosidase mRNA were detected when the carbon source was either sucrose, glucose, or fructose. These findings on the RNA level support analyses of the enzyme activity in cell suspension cultures from sycamore (Copping and Street, 1972) and carrots (Ricardo et al., 1972) grown on different carbohydrates. In contrast, expression of the three β-fructosidase genes with significant sequence homology with carrot cell wall β-fructosidase (levanase and sucrose from *B. subtilis* and invertase from yeast) is repressed by glucose (Kunst et al., 1974; Sarokin and Carlson, 1984; Martin et al., 1987). In yeast, for example, the level of invertase mRNA increased 100-fold when cells were grown on sucrose instead of on glucose.

Regulation by Wounding and Infection

In maturing carrot roots involved in sucrose storage, neither cell wall β-fructosidase mRNA nor cell wall β-fructosidase activity was detectable. Our data indicate that wounding and infection of maturing roots cause a marked, transient accumulation of β-fructosidase transcripts, followed by a transient increase in enzyme activity. Thus, apoplastic β-fructosidase is probably a new and hitherto unrecognized pathogenesis-related protein (Van Loon, 1985). The time course of the wound-induced accumulation of β-fructosidase mRNA was compared with the accumulation of mRNA coding for PAL and HRGP, two well-characterized wound-inducible and pathogen-inducible polypeptides (see, for example, Corbin et al., 1987; Lois et al., 1989). Three distinct kinetics were obtained, with the accumulation of β-fructosidase mRNA being slower than the accumulation of PAL mRNA and being faster than the accumulation of HRGP mRNA. The expression of a housekeeping gene (actin) was not affected by wounding or by infection.

A striking feature of the kinetic analysis of β-fructosidase mRNA accumulation induced by wounding or by infection was the times needed for maximal mRNA accumulation. They differed by an order of magnitude, with the induction by infection being much faster and more transient than the induction by wounding. This time difference may reflect differences in the regulation of the β-fructosidase gene. Different *trans*-acting factors could be induced by different environmental stimuli with different induction kinetics. Each of these factors could bind to an independent *cis*-regulatory element of the same gene, resulting in different transcription rates. Alternatively, several distinct signal transduction pathways may exist that are differentially triggered by wounding or by infection (Corbin et al., 1987). These different stress signal pathways may differ with respect to their transduction speeds, giving rise to the different mRNA accumulation time courses observed.

Why Do Wounding and Infection Induce Extracellular β-Fructosidase ?

The distinct kinetics of the wound-induced and pathogen-induced accumulation of the mRNAs indicate high specificity in the cellular responses to environmental stress, although a general increase in the respiration activity in response to wounding or infection in various plant storage tissues is known (Matsushita and Uritani, 1974). In root tissue of sweet potato, respiratory activity doubles within 20 hr after wounding (Greksak et al., 1972). The increased respiratory activity is paralleled by an increase in RNA content (Nawa et al., 1970) and the de novo synthesis of enzymes (Minamikawa and Uritani, 1965; Shannon et al., 1971). Hence, it is very likely that the activities of enzymes involved in the breakdown of carbohydrate reserves in-

crease at the same time. This may explain the increase in acidic, soluble β-fructosidase observed in carrot roots after wounding. This β-fructosidase may be involved in the mobilization of sucrose stored in vacuoles.

The presence of extracellular β-fructosidase in a tissue is correlated with its growth and development. In growing plant organs, the high demand of metabolically active sink cells for hexoses is satisfied by sucrose hydrolysis by an extracellular β-fructosidase. When organ growth stops, the activity of the extracellular β-fructosidase decreases and finally disappears. Cells in carbohydrate storing tissue now take up sucrose without prior hydrolysis. Thus, the decrease in activity of extracellular β-fructosidase is correlated with a switch of the metabolic activities of a tissue from growth and development to maintenance and storage of reserves independent of existing cellular sucrose uptake systems. In stress situations, cells have a high demand for hexoses to fulfill the energy and carbon requirements needed for adequate responses. This may explain the expression of the extracellular β-fructosidase in maturing storage tissue shortly after wounding or infection, which switches the cells back from a less active to a highly alert state. This response to stress observed in storage tissues may be of a more general nature, indicated by the induced expression of extracellular β-fructosidase in carrot leaves shortly after contact with a pathogen. In stressed leaves, the extracellular β-fructosidase may serve the same function as in stressed roots.

Additionally, in analogy to plant chitinases and plant β-glucanases, which seem to be directly involved in plant defense by hydrolyzing cell-surface carbohydrates (chitins and β-glucans) of fungal pathogens (Mauch et al., 1988), carrot cell wall β-fructosidase may participate in a similar defense response. Bacterial plant pathogens like *Pseudomonas* (Gross and Rudolph, 1987) or *Erwinia* (Bennett and Billing, 1980) are coated with a slime consisting of alginate and levan. The homology of the extracellular carrot β-fructosidase to the levan hydrolyzing enzyme levanase suggests that the carrot enzyme may hydrolyze the bacterial slime coat, thereby inhibiting bacterial growth directly or making the pathogen susceptible to further defense reactions. Although we did not observe levan hydrolysis in vitro, it is possible that a more sensitive assay would show that β-fructosidase is capable of hydrolyzing levans and plays a defense role in vivo.

METHODS

Plant Material and Cell Culture

Carrots (variety Nantaise) were grown from April to October in a garden near Basel, Switzerland. The carrot plants used were maturing and did not show signs of senescence. According to Ricardo and Sovia (1974), carrot tap roots from this developmental stage (about 150 days after germination) show no more significant change in root diameter but are still accumulating sucrose.

Cells of *Daucus carota*, Queen Anne's lace (wild carrot cell culture line W001C; Sung, 1976), were grown in Murashige and Skoog medium (MS medium; Murashige and Skoog, 1962) supplemented with 0.1 mg/L 2,4-D at 26°C in the dark. The suspension cells were transferred at 1-week intervals into fresh MS medium. For labeling experiments, the cells were harvested and washed with medium containing no sucrose. Labeling was done at a high cell density (1 g of cells per 15 mL of medium containing no sucrose) with D-6-^{3}H-glucosamine hydrochloride of high specific activity.

Wounding and Infecting of Carrots

Aging of carrot discs was performed as described by Chrispeels et al. (1974). Briefly, small discs (1-mm thickness, 8-mm diameter) of carrot phloem parenchyma tissue from maturing carrot roots were incubated in water on a rotary shaker at room temperature in the dark. The discs were thoroughly washed with water immediately after cutting. The water was replaced every 3 hr during the first 12 hr of the experiment and then every 12 hr until the end of the aging period. Tissue (20 g) was harvested 1 hr, 3 hr, 6 hr, 12 hr, 24 hr, 36 hr, 48 hr, and 72 hr after wounding and was immediately frozen in liquid nitrogen. Control tissue (timepoint 0) was taken directly after cutting.

A carrot with severe symptoms of the soft rot disease was used to isolate a strain of *Erwinia carotovora*. Briefly, some infection sap was plated on agarose containing LB medium (Maniatis et al., 1982) and incubated for 2 days at 26°C. For the infection experiments, the yellowish bacteria were grown overnight at 37°C in 1 mL of LB medium. The cells were spun down and resuspended in 50 mL of water. The bacterial suspension was now sprayed with a scent sprayer on the carrot plants until their surface was uniformly moistened. The infected plants were kept in glass cylinders at 26°C before the upper 2 mm of tissue was peeled directly into liquid nitrogen. Control plant tissue (timepoint 0) was directly frozen after spraying. At the same time, the leaves plus stems were harvested and frozen. Carrot plants for the infection experiment were harvested shortly before usage and rinsed with distilled water to remove the soil. Six unwounded plants per timepoint were selected and kept between moist paper towels before they were sprayed with the pathogen.

Protein Purification and Analyses

Cell wall β-fructosidase was isolated from carrot cell suspension cells as described by Laurière et al. (1988). Briefly, cell walls were prepared and extracted with 1 M sodium chloride in water. β-Fructosidase was purified by successive chromatography on CM-Sephadex, Sephacryl, DEAE-cellulose, and octyl-agarose. Tryptic glycopeptides were isolated from 0.5 mg of purified β-fructosidase labeled with ^{3}H-glucosamine as described by Sturm and Chrispeels (1986). Amino acid sequencing was done by automated Edman degradation (Edman and Begg, 1967). Polyclonal antibodies were raised against the purified protein after deglycosylation with trifluoromethanesulfonic acid (Edge et al., 1981). Specific IgG were isolated on a column of β-fructosidase linked to Sepharose.

β-Fructosidase activity was assayed at 37°C on 50 mM sucrose or raffinose in 13.5 mM citric acid and 26.5 mM sodium phosphate,

pH 4.6. The reaction was stopped with the alkaline copper reagent by Somogyi and the liberated sugars were assayed (Nelson, 1944; Somogyi, 1952). The effect of *p*-hydroxymercurybenzoate on the β-fructosidase activity was analyzed in the concentration range of 1 nM to 1 μM inhibitor.

For the separation of carrot disc proteins into soluble and cell wall-bound proteins, the tissue was ground in liquid nitrogen to a fine powder. The powder was suspended in 20 mL of cold sodium acetate buffer, pH 5.2, and placed on ice for 20 min. The homogenates were centrifuged for 30 min at 20,000 rpm and were separated into supernatants and pellets. The pellets were reextracted with 10 mL of acetate buffer. The combined supernatants were brought to 90% saturation with ammonium sulfate, and the precipitated protein was collected by centrifugation at 20,000 rpm for 30 min. Each pellet containing the soluble carrot disc proteins was dissolved in 2 mL of acetate buffer and dialyzed for 1 hr against the same buffer. The pellets containing the cell walls were washed four times with 20 mL of water and extracted overnight on ice with 10 mL of 1 M sodium chloride. Cell walls were separated from the solubilized cell wall proteins by centrifugation at 20,000 rpm for 30 min.

cDNA Synthesis and Screening Procedures

Total RNA was prepared as described by Prescott and Martin (1987) and was purified by successive lithium chloride precipitations. Poly(A^+) RNA was isolated by oligo(dT)-cellulose chromatography (Maniatis et al., 1982) and was used for cDNA synthesis. After addition of EcoRI linkers, the cDNA was size fractionated on a 0.7% agarose gel. Electroeluted cDNA with a length of 1100 bp to 2300 bp was ligated into the λgt11 vector and packaged into phages [1.2×10^6 plaque-forming units per 5 μg of poly(A^+) RNA]. The nonamplified expression library was screened with affinity-purified anti-β-fructosidase IgG according to Young and Davis (1983). One positive clone per 20,000 plaque-forming units was obtained. Twelve antibody-positive clones were rescreened with a synthetic oligonucleotide (17-mer, 96-fold degenerated) generated from the tryptic peptide sequence Tyr-Ile-Pro-Asp-Thr (5′-GT[A,G]TT[A,G]TC[A,C,G,T]GG [A,G,T]AT[A,G]TA-3′). Briefly, 1 μg of purified λ DNA was cut with EcoRI and separated on a 1.5% agarose gel. The DNA was transferred to a nylon membrane (Hybond-N, Amersham Corp.) and probed with the end-labeled oligonucleotide at room temperature. Prehybridization was done at 22°C in 6 × SSC (Maniatis et al., 1982) containing 0.25% nonfat milk powder. Hybridization was carried out in the same buffer overnight at 22°C. The DNA gel blot was washed with prehybridization buffer. The washing temperature was successively increased by 4°C increments. One positive clone was obtained. Both DNA strands were sequenced by the dideoxynucleotide chain-termination method (Messing, 1983).

Analyses of RNA and DNA

For RNA gel blot analyses, total RNA (10 μg per lane) or poly(A^+) RNA (5 μg per lane) was separated on agarose gels containing formaldehyde (Mainatis et al., 1982). RNA and DNA gel blot analyses were performed on nylon membranes (Hybond-N, Amersham Corp.) with probes labeled by random priming (Maniatis et al., 1982). Unless otherwise stated, the 5′ half of the β-fructosidase cDNA (EcoRI/EcoRI, 1100 bp) was used as a probe. Unless otherwise stated, prehybridizations were done at 65°C in 6 × SSC, 5 × Denhardt's solution, 0.5% SDS, and 100 μg/mL denatured calf thymus DNA. Hybridizations were carried out in the same buffer overnight at 65°C. The blots were washed twice with 0.1 × SSC and 0.5% SDS at 65°C for 30 min. For the gene reconstruction experiment, carrot genomic DNA was restricted with EcoRI and purified β-fructosidase cDNA titered at 0.5-copy, 1.0-copy, 2.0-copy, 3.0-copy, and 5.0-copy equivalents per carrot haploid genome (1.4×10^9 bp per haploid genome; Bennett and Smith, 1976).

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