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Normal starch content and composition in tubers of antisense potato plants lacking D-enzyme (4-α-glucanotransferase)

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Abstract. Transgenic potato (*Solanum tuberosum* L.) plants were created with sense and antisense copies of the potato D-enzyme (disproportionating enzyme; EC 2.4.1.25) cDNA linked to patatin and cauliflower mosaic virus 35 S promoters, and screened for D-enzyme activity in tubers. Transformants with sense constructs mostly had wild type D-enzyme activity but two plants had only about 1% wild-type activity. Transformants with antisense constructs had activity ranging from 90% to about 1% of wild type. Three 35 S antisense plants with very low activity were analysed in detail. Western blot analysis showed that D-enzyme was present in greatly reduced amounts in tubers and in leaves, whereas plastidic starch phosphorylase (EC 2.4.1.1) was unaffected. The lack of D-enzyme resulted in slow plant growth but development was otherwise apparently normal. Furthermore, the starch content of tubers was not appreciably altered in amount, proportion of amylose, molecular weight of debranched amylopectin, or branch chain length, despite the lack of D-enzyme. These results do not indicate a direct requirement for D-enzyme in the synthesis and accumulation of storage starch in tubers. The results are discussed in terms of the known reactions catalysed by D-enzyme and possible involvement of D-enzyme in starch metabolism.

Key words: Antisense inhibition – D-enzyme – Glucanotransferase – *Solanum tuberosum* – Starch metabolism

Introduction

D-enzyme (disproportionating enzyme or 4-α-glucanotransferase; EC 2.4.1.25) was originally found in potato tubers and shown to catalyse the disproportionation of added maltooligosaccharides by inter-molecular transglycosylation (Peat et al. 1956). Studies of the in-vitro action of D-enzyme showed that maltooligosaccharides are effective donors, that the smallest donor molecule is maltotriose, and that maltooligosaccharides and glucose serve as acceptors. A maltosyl group was the major unit transferred from donor to acceptor (Jones and Whelan 1969). However, it was recently found that D-enzyme can use high-molecular-weight starch as both donor and acceptor molecule and can catalyse the transfer of long α-1,4-glucan chains (Takaha et al. 1996), or even highly branched cluster units of amylopectin (Takaha 1996). Furthermore D-enzyme catalyses the intra-molecular transglycosylation (cyclisation) of amylose to form novel cyclic α-1,4-glucans with degree of polymerization (DP) ranging from 17 to several hundred (Takaha et al. 1996) and with amylopectin it forms cyclic glucans with a highly branched structure (Takaha 1996). In spite of the extensive characterisation of its activity in vitro, nothing is known about the function of the enzyme in vivo. It has been found in organs of numerous plants (Lin and Preiss 1988). Its wide distribution in plants, its relatively high activity and localization in plastids (Kakefuda et al. 1986; Lin et al. 1988) suggest an important role for this enzyme in starch metabolism.

To investigate the function of enzymes in starch metabolism, the antisense gene procedure has been successfully employed to inhibit the expression of genes for ADP-glucose pyrophosphorylase B subunit (Müller-Röber et al. 1992), granule-bound starch synthase (Visser et al. 1991), branching enzyme (Flipse et al. 1996), starch phosphorylase (Sonnewald et al. 1995) and soluble starch synthase (Marshall et al. 1996) in potato plants. Two such studies have clearly demonstrated the importance of these enzymes in tuber starch synthesis (Müller-Röber et al. 1992; Visser et al. 1991). In other studies, effects have been observed upon tuber starch

Abbreviations: D-enzyme = disproportionating enzyme (4-α-glucanotransferase; EC 2.4.1.25); PSP = plastidic starch phosphorylase (α-1,4-glucan phosphorylase; EC 2.4.1.1)

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appearance (Marshall et al. 1996) or physico-chemical properties (Flipse et al. 1996), but the underlying biochemical changes have not been defined. However, no changes in starch were observed after antisense inhibition of one plastidic starch phosphorylase (PSP) isoform (Sonnewald et al. 1995). In the present study, a D-enzyme cDNA (Takaha et al. 1993) was introduced into potato plants in sense and antisense orientations with patatin and cauliflower mosaic virus (CaMV) 35 S promoters, and transgenic plants with reduced D-enzyme activity were created. The effects of reduced D-enzyme activity on plant growth and starch accumulation were investigated.

Materials and methods

Plant materials. *Solanum tuberosum* L. (cv. May Queen) was obtained from the Scottish Office Agriculture and Fisheries Department, Edinburgh. Plants were grown in compost either in a glasshouse or in a growth cabinet at 25 °C with 16-h photoperiod at an irradiance of 100 $\mu mol \cdot m^{-2} \cdot s^{-1}$

Construction of vectors for transformation. Recombinant plasmids for plant transformation were constructed in *Escherichia coli* strain XL1-Blue (Stratagene, Cambridge, UK). To construct pBIC-102F, the *Sma* I-*Sac* I fragment (containing D-enzyme cDNA) of pDPE-102P (Takaha et al. 1993) was introduced into the same restriction sites of pBI-221 (Clontech, Laboratories, Basingstoke, Hampshire, UK). The *Hin*d III-*Sac* I fragment (containing CaMV 35 S promoter and D-enzyme cDNA) of this resulting plasmid was then introduced into the same restriction sites of pBI-101 (Clontech). Three other vectors were constructed as follows: pBIC-102R, same as described for pBIC-102F but pDPE-102 N (Takaha et al. 1993) was used instead of pDPE-102P; pBIP-102F, same as pBIC-102F but pUC-PAT (Bevan et al. 1986) was used instead of pBI-221; pBIP-102R, same as pBIC-102F but pDPE-102 N and pUC-PAT were used instead of pDPE-102P and PBI-221, respectively. Conjugation of these plasmids into *Agrobacterium tumefaciens* LBA4404 involved a triparental mating of the donor and host strains with *E. coli* strain HB101 carrying helper plasmid, pRK2013 (Draper et al. 1988).

Transformation of potato plants. Internodal stem sections of in-vitro-grown shoots were incubated with the recombinant *Agrobacterium* cells in 20 ml Murashige and Skoog (1962) medium (MS medium) containing 3% (w/v) sucrose for 15 min with gentle shaking at room temperature in the dark. Infected stem pieces were incubated on callus induction medium ($4.7 g \cdot l^{-1}$ MS salts, $30 g \cdot l^{-1}$ sucrose, $2 mg \cdot l^{-1}$ zeatin riboside, $200 mg \cdot l^{-1}$ NAA, $20 mg \cdot l^{-1}$ GA_3 and $8 g \cdot l^{-1}$ bacto-agar, pH 5.7) for 48 h in the dark at 20 °C. Stem pieces were then transferred onto callus induction medium containing $250 mg \cdot l^{-1}$ carbenicillin. After incubation for 4 d in the light, stem pieces were transferred to callus induction medium containing $250 mg \cdot l^{-1}$ carbenicillin and $50 mg \cdot l^{-1}$ kanamycin, and subcultured every two weeks. Stem pieces which produced callus were transferred to shooting medium ($4.7 g \cdot l^{-1}$ MS salts, $30 g \cdot l^{-1}$ sucrose, $2 mg \cdot l^{-1}$ zeatin riboside, $20 mg \cdot l^{-1}$ NAA, $20 mg \cdot l^{-1}$ GA_3 and $8 g \cdot l^{-1}$ bacto-agar, pH 5.7) containing $250 mg \cdot l^{-1}$ carbenicillin and $50 mg \cdot l^{-1}$ kanamycin, and incubated under the same conditions. Shoots were rooted twice on rooting medium ($4.7 g \cdot l^{-1}$ MS salts, $10 g \cdot l^{-1}$ sucrose and $7 g \cdot l^{-1}$ bacto-agar, pH 5.7) containing $250 mg \cdot l^{-1}$ carbenicillin and $50 mg \cdot l^{-1}$ kanamycin to confirm their kanamycin resistance.

Small-scale preparation of total protein for enzyme assays. Frozen tissue (1 g) of transgenic tubers was ground in a mortar and pestle under liquid nitrogen, and transferred to a centrifuge tube. One milliliter of extraction buffer (20 mM Tris-Cl, pH 7.5; 5 mM 2-mercaptoethanol) was added, vortexed, then centrifuged at 4 °C at 11 000 g for 5 min. To remove the sugars in the homogenate, 500 µl of supernatant was loaded onto a PD-10 column (Pharmacia, Milton Keynes, UK) and then eluted into 1.2 ml of 100 mM Tris buffer (pH 7.0). This eluate was used for protein assay, D-enzyme activity assay (Takaha et al. 1993), activity detection of phosphorylase isozymes after native PAGE (Steup 1990), and immunodetection of D-enzyme protein after SDS-PAGE (Harlow and Lane 1988). One unit of D-enzyme activity is defined as the amount which produces 1 µmol glucose $\cdot$ min^{-1}.

Production of anti-D-enzyme antisera. D-enzyme polypeptide was expressed as a fusion protein with *E. coli* glutathione transferase (GST). The *Xho* I-*Hin*d III fragment of pDPE102P, encoding the C-terminal 370 amino acids of D-enzyme was introduced into pGEX-3X (Pharmacia) and pGEX-301DL produced. The *E. coli* cells carrying the plasmid pGEX-301DL were cultured in LB medium (Sambrook et al. 1989) at 37 °C until late logarithmic phase, then isopropyl β-D-thiogalactoside (2 mM) was added and incubated for 3 h at 37 °C. Inclusion bodies were isolated and separated by SDS-PAGE and the fusion protein was excised, electro-eluted, and used as an antigen for immunization of rabbits.

Quantitation of total starch in potato tubers. Frozen tuber tissue (1 g) was powdered and transferred to a centrifuge tube containing 5 ml of 80% (v/v) ethanol and incubated at 80 °C for 30 min with occassional vortexing, then centrifuged at 4000 g for 15 min and the supernatant discarded. Washing of the pellet with 80% (v/v) ethanol was repeated at 80° C, and centrifuged. The pellet was solubilized in 12 ml dimethylsulfoxide (DMSO) and boiled for 30 min. After cooling, 3 ml of 8 N HCl was added, the solution was incubated for a further 30 min at 60 °C, and then left overnight at room temperature. The sample was then centrifuged at 4000 *g* for 15 min, and the starch was precipitated with ethanol. The starch pellet was solubilised in DMSO, then a portion was treated with glucoamylase and α-amylase, and the released glucose was measured by the glucose oxidase method (Miwa et al. 1972).

Purification and analysis of starch granules from tubers. Starch granules (400 mg) prepared as by Vos-Scheperkeuter et al. (1986) were solubilised in 40 ml of 90% (v/v) DMSO. For debranching, the starch solution (500 µl) was mixed with 4.5 ml of 20 mM sodium acetate buffer (pH 3.5) and 1 unit of isoamylase (Hayashibara Biochemical Lab., Okayama, Japan) and incubated overnight at 40 °C. The debranched glucan was recovered as a dry pellet after ethanol precipitation, dissolved in 75 µl of 1 N NaOH, then analysed by high-performance anion-exchange chromatography (HPAEC) or by gel-filtration chromatography using a Superdex 75 column (Pharmacia; 300 mm long, 10 mm i.d.) as described previously (Takaha et al. 1996). Estimation of amylose content was carried out by the method of Hovenkamp-Hermelink et al. (1988).

Results

Production of transgenic potato plants with altered levels of D-enzyme activity. In order to obtain potato plants with altered levels of D-enzyme, four gene vectors for Ti-plasmid-mediated transformation were constructed (Fig. 1). pBIC-102F and pBIC-102R were designed to synthesize sense and antisense RNA, respectively, from D-enzyme cDNA under the control of the CaMV 35 S promoter. pBIP-102F and pBIP-102R were designed to synthesize sense and antisense RNA, respectively from D-enzyme cDNA under the control of the patatin promoter. Stem cuttings of potato, cv. May Queen,

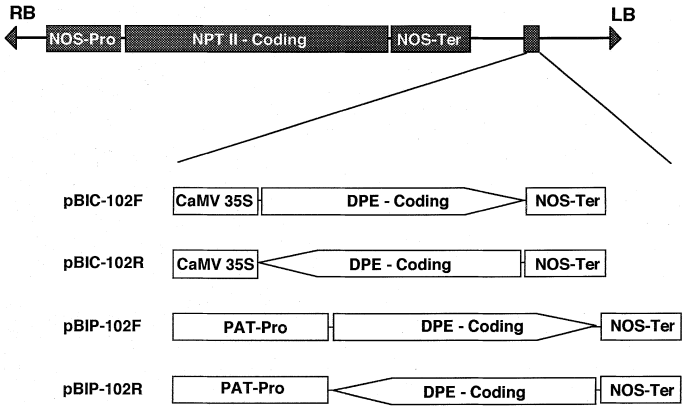

Fig. 1. Structures of plant transformation vectors. Plant transformation vectors were constructed as described in Materials and methods. *RB*, T-DNA right border; *LB*, T-DNA left border; *NOS-Pro*, nopaline synthase gene promoter sequence; *NOS-Ter*, nopaline synthase gene terminator sequence; *NPT II-Coding*, neomycin phosphotransferase-II coding sequence from Tn5; *CaMV 35 S*, cauliflower mosaic virus 35 S promoter sequence; *DPE-Coding*, D-enzyme coding sequence; *PAT-Pro*, patatin promoter sequence. The DPE-coding sequence is inserted either in forward (*F*) or reverse (*R*) orientation to give sense and antisense expression, respectively

were transformed with these gene constructs and regenerated shoots were rooted twice on medium containing kanamycin to confirm their kanamycin resistance. Selected regenerated plants were tested by polymerase chain reaction (PCR) analysis to establish that rooting on kanamycin medium was a reliable means of identifying transformants (results not shown). Regenerated transformants (T1–1 plants) were then transferred to soil and grown in the greenhouse and tubers were harvested (T1–1 tubers).

One tuber for each independent transformant was used to examine D-enzyme activity. Results (Fig. 2) show reduced D-enzyme activity (from 90 to 1% of wild type) in transgenic plants carrying antisense constructs. Greater reduction of D-enzyme activity was achieved with the 35 S promoter than with the patatin promoter. D-enzyme activities of most of the sense transformants were not much changed from wild type (range within ±20% of wild type). However two sense transformants (D103 and D204) showed greatly reduced D-enzyme activity (about 1% of wild type).

Three antisense transformants with reduced D-enzyme activity (E105, E211 and E209) and the wild type were used for further investigation. These transformants all contained the 35 S promoter. Tuber and leaf extracts from each plant were subjected to western blot analysis using an antibody for D-enzyme. The strong signal found in wild-type tubers could not be detected in the tuber extracts of E211 or E209, but a very faint band was detected in the E105 extract (Fig. 3A, DPE). For comparison, the amount of PSP was examined, since this enzyme may function in conjunction with D-enzyme (Takaha et al. 1993). Activity staining after PAGE showed a constant amount of PSP in wild-type and antisense plants, both in tubers (Fig. 3A) and leaves (Fig. 3B).

Stability of low D-enzyme activity in transgenic plants. The three transgenic plants (E105, E209, E211) and wild type were maintained by in-vitro shoot culture and propagated by stem cuttings. In order to obtain tubers for further analysis, four in-vitro shoots for each were transferred to soil and grown in pots in a greenhouse (T1–2 plants) and tubers harvested (T1–2 tubers). In order to examine the stability of reduced D-enzyme activity in the second generation, four T1–2 tubers for each construct were planted and grown in the greenhouse and tubers harvested (T2–1 tubers). D-enzyme activities in these tubers are summarized in Table 1. E211 and E209 showed very low (less than 2% of wild type) D-enzyme activity in tubers produced from in-vitro shoots (T1–2), and in the tubers in the second generation (T2–1 tubers). Thus the suppression of D-enzyme gene expression and the resultant low D-enzyme activity seems to be stable in these two transgenic lines. The D-enzyme activity in E105 was not as low as E211 and

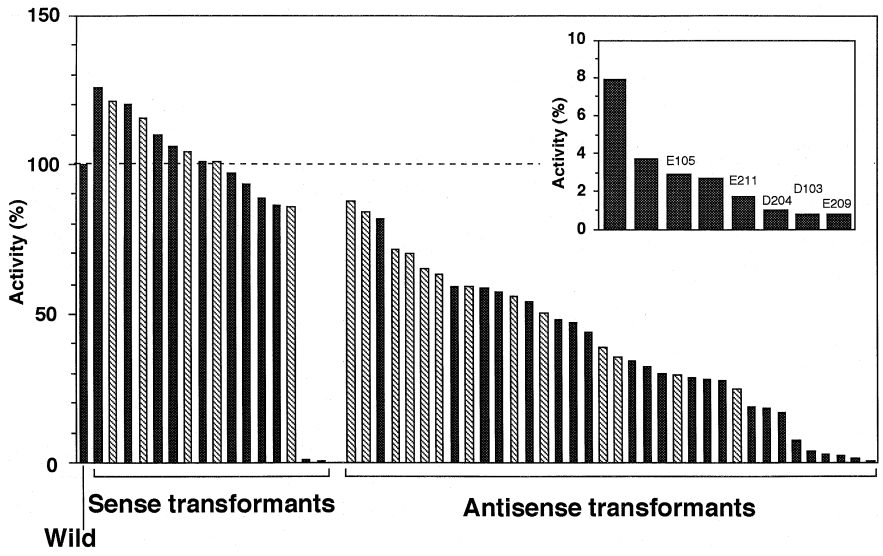

Fig. 2. D-enzyme activities in tubers of transformed potato plants. One tuber from each independent transformant was used to examine the D-enzyme activity. D-enzyme activities are expressed relative to that of wild type [0.385 ± 0.027 units · (mg protein)$^{-1}$]. *Solid* and *hatched bars* are plants transformed with 35 S promoter and patatin promoter constructs, respectively. The box (*inset*) gives values for selected antisense transformants (E105, E211 and E209) and two sense transformants (D103 and D204) with very low activity

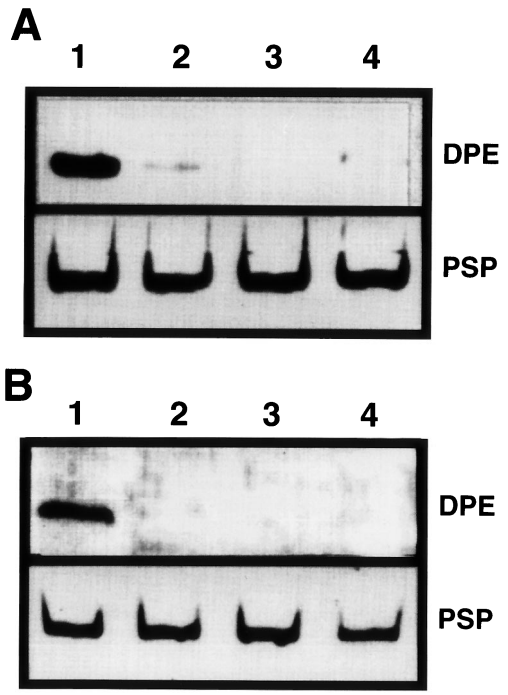

Fig. 3A, B. Inhibition of D-enzyme synthesis in tubers (**A**) and leaves (**B**) of transgenic plants. Total protein (20 μg and 70 μg for tubers and leaves, respectively) from each was separated by SDS-PAGE, blotted onto membrane and probed with D-enzyme antiserum (*DPE*). Total protein (20 μg and 90 μg for tubers and leaves, respectively) from each was separated by native-PAGE containing glycogen and plastidic starch phosphorylase activity is visualized as described in *Materials and methods* (*PSP*). *Lane 1*, wild type; *lane 2*, E105; *lane 3*, E211; *lane 4*, E209

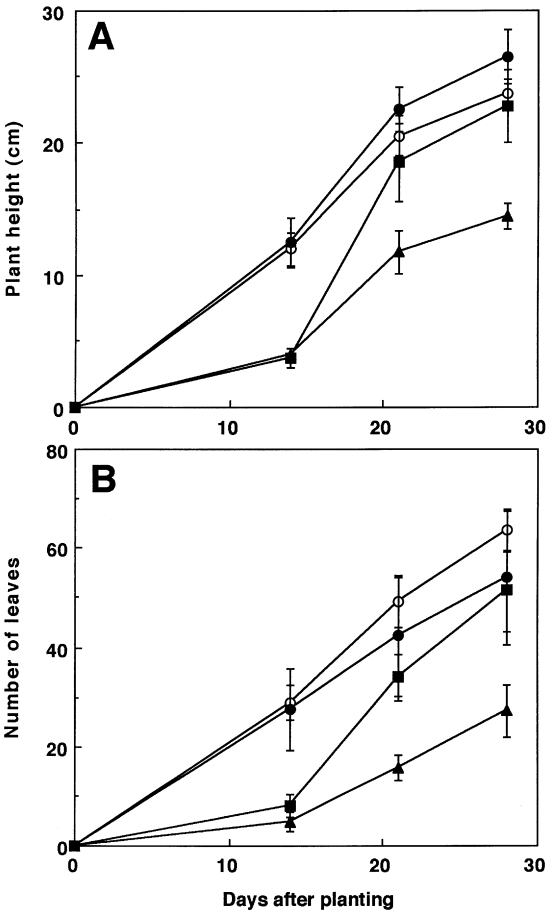

Fig. 4A,B. Effect of decreased D-enzyme activity on plant height (**A**) and number of leaves (**B**). T1–2 tubers were planted in pots and incubated in the growth room at 25 °C with a 16-h photoperiod at an irradiance of 100 μmol · m^{-2} · s^{-1}. The height of the plants (**A**) and the number of leaves (**B**) were measured during the initial few weeks. Value are the mean ± SE of four plants for each point. ○, wild type; ●, E105; ■, E211; ▲, E209

E209, and ranged between 3 and 20%. However, in all cases, the order of D-enzyme activity was maintained as E209 < E211 < E105.

Effect of reduced D-enzyme activity on plant growth and tuber development. The effect of reduced D-enzyme activity on plant growth and development was investigated. It was apparent that tubers with the lowest D-enzyme activity produced sprouts more slowly than wild-type tubers (results not shown). Therefore, to compare the growth of plants, four T1–2 tubers for each transformant were kept in the dark at room temperature until they produced sprouts, then kept in the cold until all tubers had produced strong sprouts. All the sprouts were removed from the seed tubers except the strongest one, then tubers were planted in 25-cm pots and maintained in the growth room. The height of the plants and the number of leaves were measured during the first few weeks (Fig. 4). The height and leaf number of E209 plants were significantly less than those of the wild type at all points tested, and E211 plants also apparently grew slower than the wild type. These plants were subsequently transferred to the greenhouse and grown to maturity. The number of tubers (more than 2 cm in diameter) and total weight of tubers from each plant were measured (T2–1 tubers). No significant difference was observed in

Table 1. D-enzyme activity in tubers from wild-type (WT) and transgenic potato plants

Transformant	T1-1		T1-2		T2-1	
	Activity	%	Activity	%	Activity	%
WT	0.360 ± 0.017	100	0.368 ± 0.025	100	0.348 ± 0.037	100
E105	0.011 ± 0.004	3.1	0.016 ± 0.010	4.3	0.071 ± 0.049	20.4
E211	0.006 ± 0.006	1.7	0.005 ± 0.002	1.4	0.006 ± 0.004	1.7
E209	0.003 ± 0.001	0.8	0.005 ± 0.002	1.4	0.004 ± 0.002	1.1

Values are the means obtained from at least three independent plants. D-enzyme activity is expressed as units · (mg protein)$^{-1}$ ± SD. Percentages are expressed relative to wild-type values

Table 2. Numbers and total weight of tubers from wild-type (WT) and transgenic potato plants

Transformant	Number of tubers	Total weight of tubers	
		(g)	(%)
WT	14.0 ± 3.0	420.0 ± 122.0	100
E105	10.8 ± 1.3	422.5 ± 55.3	100.6
E211	11.8 ± 5.0	318.6 ± 39.2	75.9
E209	12.8 ± 5.6	297.4 ± 13.9	70.8

The value is the mean of at least three independent plants

Table 3. Starch and amylose content in tubers of wild-type (WT) and transgenic potato tubers

Transformant	Starch content $\mu g \cdot (g\ FW)^{-1}$	Amylose content (%)
WT	73.8 ± 11.8	19.1 ± 1.2
E105	73.8 ± 5.6	20.7 ± 0.9
E211	85.9 ± 9.3	19.9 ± 0.8
E209	66.1 ± 6.5	19.1 ± 0.9

Assays were carried out on at least three independent plants. The values for starch content and amylose content are the mean ± SE

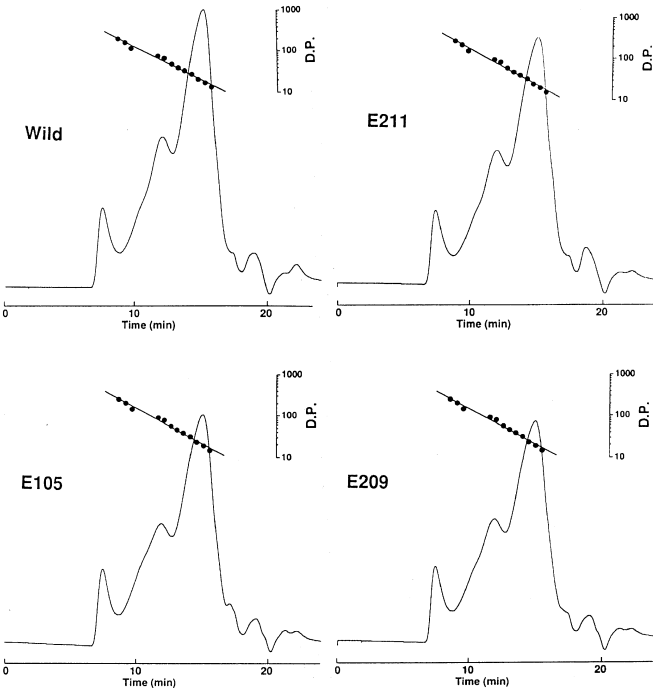

Fig. 5. Analysis of debranched starch by gel-filtration chromatography. Starch granules were prepared from tubers and solubilized in 90% (v/v) DMSO, then completely digested with debranching enzymes as described in *Materials and methods*. Debranched glucans were loaded onto a Superdex 75 (300 mm long, 10 mm i.d.) gel-filtration column with flow rate of 1 ml per min. Carbohydrates were detected with an RI detector. For the calibration of DP, the eluent was fractionated and the DP of debranched glucan in each fraction was calculated by measuring the total carbohydrate by the phenol-sulphuric acid method (Dubois et al. 1956) and reducing power by the modified Park-Johnson method (Hizukuri et al. 1981)

number of tubers between wild and transgenic plants (Table 2). However, there appeared to be a small reduction in the mass of tubers produced by E211 and E209 plants. The slow growth of antisense plants and low yield of tubers was observed on other occasions but was apparently more pronounced in plants maintained in the growth room than in those grown in the greenhouse (results not shown). Furthermore, E211 and E209 plants flowered later than the wild type and E209 flower buds often abscised before opening (results not shown). Although our results indicate that plant growth and tuber yield of antisense plants is affected by growth conditions, a systematic investigation would be required before clear conclusions can be reached.

Effect of reduced D-enzyme activity on starch in tubers. The effect of reduced D-enzyme activity on starch was investigated. The amount of starch in T1–2 tubers from the wild type and the three transgenic plants (E105, E211 and E209) was measured, but no significant difference between wild and transgenic plants was observed (Table 3). Scanning electron microscopy of sections of tubers showed that starch grain sizes and numbers per cell were indistinguishable from the wild type, and observation of grains by phase-contrast microscopy and staining with iodine failed to detect any differences in appearance (results not shown). Starch granules were then purified from T1–2 tubers of wild-type and transgenic plants, to investigate starch structure. The amylose content was analysed, but no difference was found between wild-type and transgenic plants (Table 3). The starch from each plant was then debranched completely by isoamylase treatment, and the debranched amylopectin was analysed by gel-filtration chromatography (Fig. 5) and by high-performance anion-exchange chromatography (Fig. 6). However, no appreciable difference was found between wild-type and transgenic plants, in the molecular weight of the debranched amylopectin or branch chain lengths. Measurements of the viscocity of solutions of starch from tubers suggested, in some cases, that lack of D-enzyme correlated with reduced viscocity (results not shown). Furthermore, small-angle X-ray scattering measurements suggested some changes in starch grain crystalinity (T. Waigh and A. Donald, University of Cambridge, personal communication). However, such differences in physico-chemical properties of the starch were not consistent, and seemed to reflect the vigour of plant growth (i.e. they were more apparent in plants grown in the growth room, than in those grown in the greenhouse). Thus it was concluded that changes in the physical properties of the starch in antisense plants could be an effect of slow plant growth rather than a direct effect of lack of D-enzyme. Therefore, further detailed studies of starch structure and physical properties were not undertaken.

Discussion

Introduction of antisense genes successfully produced transgenic plants with decreased D-enzyme activity,

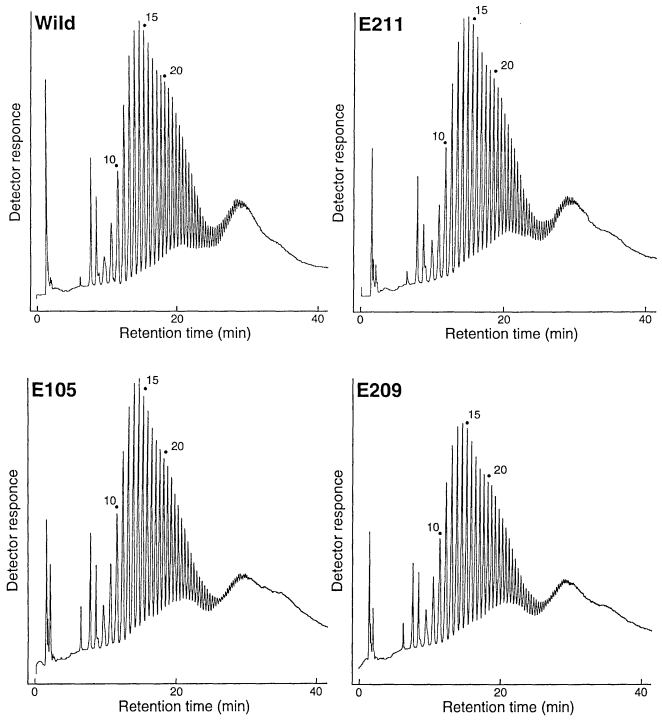

Fig. 6. Analysis of debranched starch by high-performance anion-exchange chromatography (HPAEC). Debranched glucans prepared for the analysis in Fig. 6 were analysed by HPAEC with a pulsed amperometric detector. Conditions for HPAEC are described in *Materials and methods*. Retention times for glucans with DPs of 10, 15 and 20 are indicated

both in tubers and in leaves, and two of the plants had only about 1% of wild-type D-enzyme activity in tubers. Various phenotypic changes were observed in these transgenic plants (E211 and E209); however, they were most evident in E209, the plant with the lowest D-enzyme activity. The E209 plants grew more slowly than the wild type (Fig. 4A) and produced fewer leaves (Fig. 4B) when they were grown in the growth room. The E209 plants also produced a lower tuber mass (Table 2). These phenotypic changes also seemed to be found in another transformant, E211, which had the second lowest activity, but were not so evident in E105. Thus, these phenotypic changes correlated well with their D-enzyme activity. Furthermore, similar phenotypes were also found in sense transformants having very low (about 1% of wild type) D-enzyme activity (results not shown), but were not found in any of the other 49 sense and antisense transformants shown in Fig. 2. These results strongly suggest that these phenotypic changes are produced by decreased D-enzyme activity, but are only found when D-enzyme activity is extremely low. Although a difference was found in plant growth, the starch accumulated in tubers was not significantly altered in amount (Table 3), proportion of amylose (Table 3), or amylopectin structure (Figs. 5, 6). The amount of starch found in leaves was highly variable between plants and between different leaves on the same plant, even in the wild type (results not shown). Therefore, it was not possible to establish if lack of D-enzyme affects the accumulation or structure of leaf starch.

These results indicate that lack of D-enzyme reduces the vigour of plant growth. This is consistent with a role for D-enzyme in starch metabolism, and hence in source-sink relations, but that role remains unknown. It has been generally thought that D-enzyme is involved, together with PSP, in the complete metabolism of maltooligosaccharides during starch breakdown in the plastid. Glucans are broken down by PSP no further than maltotetraose, and it is proposed that D-enzyme converts such oligosaccharides into longer molecules which can be further attacked by PSP. A similar maltooligosaccharide utilization system exists in *E. coli* (Schwartz 1987), consisting of amylomaltase (D-enzyme equivalent) and α-1,4-glucan phosphorylase (both encoded by the mal A operon). Recent results now lead us to consider that such a maltooligosaccharide utilization system may also operate during starch synthesis, since it has been reported that maltooligosaccharides may be produced by the 'trimming' activity of debranching enzyme on the elongating amylopectin molecule (Ball et al. 1996). D-enzyme and PSP may be involved in the conversion of maltooligosaccharides, produced by the trimming activity of debranching enzyme, into glucose-1-phosphate which can be used again by ADP-glucose pyrophosphorylase for starch synthesis. Such a requirement for maltooligosaccharide recycling may explain the substrate preference of PSP for maltooligosaccharides rather than high-molecular-weight branched glucans, and the induction of D-enzyme and PSP genes during starch synthesis (Takaha et al. 1993; St-Pierre and Brisson 1995; Duwenig et al. 1997).

If this explanation of D-enzyme function is correct, it might be expected that D-enzyme antisense plants would accumulate maltotetraose in leaves and tubers, and that this may restrict the mobilization of carbohydrate from source tissue to sink tissue (from source leaf to tuber or from tuber to sprouts). We have not detected maltooligosaccharides in such tissues (results not shown) but they might be difficult to isolate and detect, or may be removed by another mechanism in vivo. Another possibility is that in the absence of D-enzyme, maltooligosaccharides are utilized by hydrolytic enzymes (α-amylases or α-glucosidases), producing glucose rather than glucose-1-phosphate. This would be energetically less favorable and could explain the slow growth phenotype of antisense D-enzyme plants. Yet another possibility is that D-enzyme is involved in the metabolism of cyclic glucans, but these have not yet been detected in vivo (Takaha et al. 1996).

In conclusion, we have established that D-enzyme does have an important role to play in plant growth and development, but that its precise function in starch metabolism cannot be determined with the plants that we have created. Any changes observed in starch structure, physico-chemical properties or turnover could be attributed to the reduced vigour of plants lacking D-enzyme. In principal, effective tuber-specific antisense inhibition using the patatin promoter would have produced plants with normal starch metabolism in

leaves and hence vigorous growth, so allowing more meaningful studies of the role of D-enzyme in tuber starch metabolism. Unfortunately the patatin promoter was appreciably less effective than the 35 S promoter (Fig. 2). In future experiments it will be necessary to devise systems in which D-enzyme activity can be regulated in more-specific ways such that its potential roles in starch synthesis and turnover can be studied separately, and independently of secondary effects upon plant growth.

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