ELSEVIER

Carbohydrate Research 275 (1995) 361–369

CARBOHYDRATE RESEARCH

Structural analysis of starch from normal and *shx* (shrunken endosperm) barley (*Hordeum vulgare* L.)

Alan H. Schulman [a,*], Shinji Tomooka [b], Ayako Suzuki [c], Päivi Myllärinen [d], Susumu Hizukuri [b]

[a] *Institute of Biotechnology, University of Helsinki, P.O. Box 45, Karvaamokuja 3a, FIN-00014 Helsinki, Finland*

[b] *Department of Biochemical Science and Technology, Faculty of Agriculture, Kagoshima University, 21-24 Korimoto 1, Kagoshima 890, Japan*

[c] *Department of Natural Sciences, Osaka Women's University, Daisen-cho 2, Sakai 590, Japan*

[d] *VTT Biotechnology and Food Research, P.O. Box 1500, FIN-02044 Helsinki, Finland*

Received 15 November 1994; accepted 31 March 1995

Abstract

The *shx* shrunken endosperm mutation of barley is the only one described with its primary effect on Type I, primer-independent soluble starch synthase (SSS). The mutant produces grains with only 31% of the normal dry weight of starch, associated with a decrease in A-granule dimensions. We investigated the structure of *shx* and normal cv. Bomi barley amylose and amylopectin. Surprisingly, both amylose and amylopectin from the two genotypes were very similar in all aspects examined. These findings are discussed in the light of the potential roles for the various forms of SSS and branching enzyme.

Keywords: Soluble starch synthase; Amylose; Amylopectin; Mutant; Barley

1. Introduction

Much information on the relationship between starch structure and functional properties and on the specific roles of the starch biosynthetic enzymes has been acquired by analysis of starch synthetic mutants, particularly in maize. The most relevant mutants in this regard are those of the starch synthases and starch branching enzymes. The linear α-(1 $\rightarrow$ 4) bonds of amylose are formed by the granule-bound starch synthases (GBSS),

* Corresponding author.

SSDI 0008-6215(95)00169-7

while the α-(1 → 4) bonds of amylopectin are synthesized by the soluble starch synthases (SSS, ADP-Glc:(1 → 4)-α-D-glucan 4-α-D-glucosyl transferase, EC 2.4.1.21 [1,2]. Two kinds of SSS have been identified, Type I, which is primer-independent in vitro, and Type II, which requires a glucan primer for in vitro activity. Both types have been reported to occur in multiple forms in a variety of plants, and we have detected in barley three electrophoretic forms for each type on native, activity-stained gels [3]. The α-(1 → 6) branch points are formed by the starch branching enzymes (SBE) in a transferase reaction involving no new synthesis.

The *wx* mutation, which destroys expression of the 60-kD GBSS I [4], eliminates amylose as well [5,6]. This mutant has served as the genetic background for study of many double and triple mutants affecting amylopectin structure [6,7]. Only two cereal mutants are known to affect SSS. The *du* (*dull*) mutant of maize affects both SBE IIa and SSS II, the latter of which is reduced to ca. 40% of normal [8]. The amylose content as measured is high in *du* [9–12], and the proportion of long ($\overline{cl} \geq 20$) B-chains is decreased as compared to short B-chains ($20 > \overline{cl} \geq 4$) [7]. Since *du* is epistatic to *ae* [9] (*amylose extender*) which likewise affects SBE IIa, the primary effect of *du* may be on Type II SSS. The only other mutant in any organism known to affect Type II SSS is *st-3* of the green alga *Chlamydomonas reinhardtii*, with a phenotype similar to *du* but with no effects on SBE [13].

The *shx* mutant of barley [14] is so far unique in its primary effect on SSS [15]. This mutation is inherited as a single-locus Mendelian recessive trait, with the phenotype of the grain independent of the maternal phenotype or the direction of the cross (xenia). At maturity, *shx* contains only 31% of the normal weight of starch per grain, with A-granules reduced in size but B-granules of normal dimensions [16]. Developing grains of *shx* contain increased levels of sucrose, hexoses and hexose phosphates, and, critically, the substrate for starch synthesis, ADP-Glc [17].

Since this change is coupled with the loss of the primer independence of one of the three Type I SSS electrophoretic forms detected [3] and a concomitant 85% reduction in total SSS I activity [15], SSS I, particularly the affected form, appears to exercise a major quantitative control over starch synthesis in barley. The importance of SSS on flux control in developing endosperm is supported by recent work with temperature effects on grain filling [18,19].

Given the importance of SSS in starch biosynthesis and its role in the catalysis of α-(1 → 4)-D-glucosyl bond formation in amylopectin biosynthesis, together with the great reduction in SSS activity in *shx* and the associated deficiency in starch biosynthesis in this mutant, we wished to determine the effects of *shx* on starch structure.

2. Experimental

Plant material.—Plants were grown and grains harvested at maturity (more than 55 days after anthesis) as described previously [15]. The *shx* mutant was studied as a nearly isogenic line (more than six backcrosses) in cv. Bomi, which served as the control. Only homozygous grains were analyzed.

Starch isolation.—Starch was isolated from grains using the method of McDonald and Stark [20] with some modifications. Barley grains were first milled gently with a

KT-mill (Koneteollisuus Ltd., Finland) and suspended in 0.02 M HCl. The separated fiber fraction and the brown, proteinaceous layer were each treated with protease (Sigma, type XIV, P-5147, from *Streptomyces griseus*). At the end of the separation, all starch fractions (5 g) were combined and treated twice with 500 mL 1:7 toluene–0.2 M NaCl, washed twice with water and twice with acetone, and finally air-dried.

Fractionation of starch.—Prior to fractionation, starch was fully defatted by dissolving in hot dimethyl sulfoxide and precipitating with ethanol twice as described by Takeda et al. [21]. The defatted starch was fractionated into amylose and amylopectin by the method of Lansky et al. [22], without autoclaving, under a stream of nitrogen to avoid oxidative degradation [21].

Analytical methods.—Iodine affinity was determined by automated [23] amperometric titration [24]. The blue value at 680 nm and λ_{max} were measured on a solution stained with iodine as described previously [25]. The amylose content was determined from the iodine affinities of starch, amylose, and amylopectin. The limiting viscosity $[\eta]$ was measured in 1 M KOH using an Ostwald viscometer at 22.5°C. Number-average chain length ($\overline{cl}_n$) was calculated from the non-reducing terminal residues, which were assayed photometrically [26] for amylopectin or fluorimetrically [21,27] for amylose after rapid Smith degradation [26]. The $\overline{cl}$ of amylopectin was also assayed by reducing value following degradation with isoamylase, which was carried out in a 0.2% (w/v) solution of *Pseudomonas* isoamylase (0.3 U/mg) for 12 h at 45° and pH 3.5 (50 mM acetate buffer). The reducing value was measured by Somogyi's method [28] using Nelson's reagent [29], but the heating period was extended to 30 min to give a constant value per mole independent of dp [30]. Carbohydrate was determined by the phenol–sulfuric acid method [31]. Beta-amylolysis was performed with a crystalline sweet-potato preparation [32] which had been further purified by recrystallization from ammonium sulfate. The photopastegraph (Hirama Rika Kenkyujo) was operated on a 0.1% starch suspension, with the temperature increased at 2°C/min, and the transmittance was measured at a wavelength of 372 nm.

The number-average degree of polymerization ($\overline{dp}_n$) was determined colorimetrically by the Park–Johnson method with modifications [27], applying the corrected procedure reported elsewhere [33] which yielded the same value per mole irrespective of molecular size. The weight-average degree of polymerization ($\overline{dp}_w$) and the dp distribution of amylose were determined by HPLC combined with a low-angle laser-light-scattering photometer (LALLS) and a differential refractometer as described elsewhere [34,35]. The chain-length distribution of amylopectin was also determined by an HPLC-LALLS system (Toso LS-8000). Columns of Asahi-pak GS320 (7.6 × 500 mm), Asahi-pak GFA30 (7.6 × 500 mm), and TSK-Gel G3000PW (7.6 × 600 mm) connected in series were used. The columns were eluted with 0.1 M sodium phosphate buffer, pH 6.1, containing 0.02% of sodium azide and 1.5% of acetonitrile with a flow rate of 0.25 mL/min.

3. Results

The iodine affinities of starch and its components from normal and *shx* cv. Bomi barley are listed in Table 1. Normal and *shx* starch have similar amylose contents,

Table 1
Iodine affinities and amylose contents of barley starches [a]

Property	Bomi	*shx*
Iodine affinity (g/100 g)		
Starch	3.91 ± 0.056	3.95 ± 0.087
Defatted starch	6.08 ± 0.09	6.07 ± 0.10
Amylose	20.2 ± 0.43	19.4 ± 0.26
Amylopectin	0.73 ± 0.006	0.82 ± 0.006
Amylose content (%)	27.5	28.2

[a] Mean value ± standard deviation, $n = 3$.

within the range for non-waxy barley observed earlier [36,37]. The iodine affinities of the starches of the two genotypes, both in defatted and non-defatted form, were very close, suggesting that both starches contain fairly similar amounts of lipid. The amylose of *shx* showed a slightly lower, and amylopectin a slightly higher, iodine affinity than that of normal cv. Bomi. To help establish whether these differences were significant, we examined the structures of these molecules in more detail.

As presented in Table 2, normal and *shx* amyloses had very similar properties in every analysis. The gel-permeation HPLC of both amyloses showed like distribution patterns, with the apparent dp distributions of normal and *shx* amyloses found to be respectively 180–16 300 and 210–17 200. These data indicate that both amyloses have very similar molecular size distributions, comparable with those of several wheat amyloses [38] and a little broader than distributions seen for rice amyloses [21,39].

The general properties of normal and *shx* amylopectins analyzed by conventional methods indicated very similar structures (Table 3). In addition, both the chain-length distributions (Fig. 1 and Table 4) by gel-permeation HPLC were almost identical. The iodine affinities were similar to those for rice amylopectins with intermediate iodine

Table 2
Properties of barley amyloses [a]

Property	Bomi	*shx*
Blue value [b]	1.4 ± 0.02	1.39 ± 0.07
Iodine coloration [b], λ_{max} (nm)	653 ± 2.6	652 ± 2.2
Limiting viscosity [c] (mL/g)	257 ± 3.8	267 ± 7.2
Number-average dp [c] ($\overline{dp}_n$)	1120 ± 20	1230 ± 27
Weight-average dp [c] ($\overline{dp}_w$)	4470 ± 147	4610 ± 162
$\overline{dp}_w/\overline{dp}_n$	4.1	3.7
Apparent dp distribution [c]	180 (±19)–16300 (±570)	210 (±61)–17200 (±95)
Number-average chain length [b]	210 ± 12	230 ± 4.9
Average number of chains [b]	5.4 ± 0.06	5.3 ± 0.23
Beta-amylolysis limit (%) [b]	87 ± 1.0	82 ± 1.6

[a] Mean value ± standard deviation.
[b] $n = 5$.
[c] $n = 3$.

Table 3
Properties of barley amylopectins [a]

Property	Bomi	*shx*
Blue value [b]	0.09 ± 0.008	0.11 ± 0.003
Iodine coloration [b], λ_{max} (nm)	540 ± 1.0	546 ± 1.6
Limiting viscosity [c] (mL/g)	147 ± 3.7	148 ± 0.9
Number-average dp [c] ($\overline{dp}_n$)	8700 ± 258	7800 ± 279
Number-average chain length Smith degradation [b]	20.2 ± 0.5	19.9 ± 0.3
Isoamylolysis [b]	19.0 ± 0.3	19.4 ± 0.4
Beta-amylolysis limit [b](%)	60 ± 0.98	59 ± 0.74

[a] Mean value ± standard deviation.
[b] $n = 5$.
[c] $n = 3$.

affinity (IR48 and IR64) [39] and to those of wheat amylopectins [38]. This property is thought to be related to the amount of the long-chain components [40] (LC_1 and LC_2, Fig. 1) according to eqn (1), below.

$$\%LC_1 + \%LC_2 = 5.4 \times \text{iodine affinity} + 0.33 \quad (1)$$

From the iodine affinity values of normal and *shx* starch, 0.73 and 0.85, this equation predicts 4.3 and 4.9% of long-chain components, respectively. We measured 4.3% in normal and 4.8% in *shx* starch, confirming the predictive value of the relationship also for barley amylopectin. These LCs are not found in waxy varieties, but are present to different extents in the amylopectins of various normal starches, such as those of rice

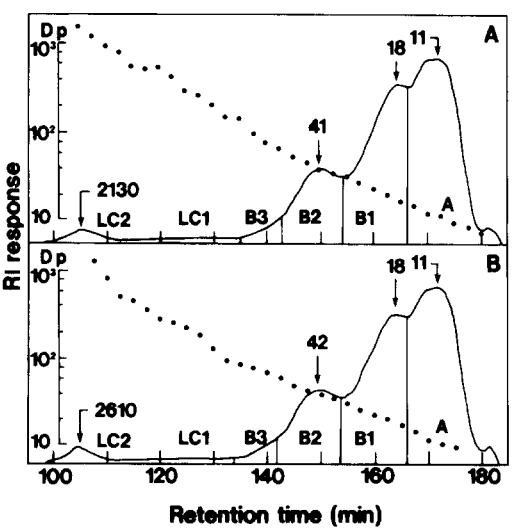

Fig. 1. Chain-length distributions of Bomi (A) and *shx* (B) amylopectins. Dp of peak apices shown as numbers with arrows; ●, dp determined directly from RI and LALLS responses.

Table 4
Distributions of the chain lengths of barley amylopectins

Fraction [a]	A	B1	B2	B3	CL1	CL2	Whole	CL1 + 2	ΣA, B1–3
Bomi									
$\overline{cl}_w$ [a]	13	23	45	69	235	2210	95	1390	23
Weight(%)	43.8	33.6	15.7	2.6	1.9	2.4	100	4.3	95.7
Mole(%)	64.47	27.95	6.68	0.73	0.15	0.02	100	0.1	99.9
shx									
$\overline{cl}_w$	13	23	45	74	234	3150	113	2240	24
Weight(%)	43.1	33.3	16.3	2.5	2.3	2.5	100	4.8	95.2
Mole(%)	64.13	28.0	7.01	0.65	0.19	0.02	100	0.2	99.8

[a] See Fig. 1.

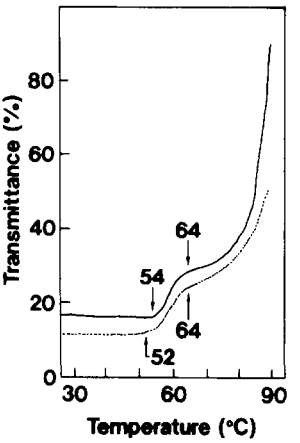

Fig. 2. Photopastegrams of Bomi (———) and *shx* (---) starches.

(denoted F1a, ref. [40]), normal maize [41], and lotus [42]. Similar distributions for barley amylopectins have been reported by MacGregor and Morgan [43]. Their peak 1 fraction, which appears to correspond to LC1 and LC2, was assumed to have a chain length of at least 60 due to its elution in the void volume from a Bio-Gel P-6 column. In our case, three tandemly linked columns resolved chain lengths over $dp \times 10^3$ and separated LC1 from LC2 well.

The pasting properties of normal and *shx* starches were examined by photopastegraphy, which is a useful method for characterizing the thermal gelatinization of starch [41]. The photopastegrams (Fig. 2) showed that both normal and *shx* starches had a very similar two-step pasting behavior, although the *shx* starch showed a slightly lower gelatinization temperature (52°) than that (54°) of normal starch. Both showed a similar stagnancy of pasting between 64° and ~ 70°, possibly due to starch lipids.

4. Discussion

No aspect of starch structure investigated differed greatly between the normal and *shx* genotypes in the cv. Bomi genetic background. We found this surprising in view of the low activity of total and Type I SSS and the reduction in total starch synthesized in *shx*. The slight increase in long chains in *shx* contrasts with the amylopectin structure observed in the *st*-3 mutation of Type II SSS in *C. reinhardtii*, where the number of intermediate-length chains (dp 8–25) decreases and the number of short chains (dp 2–7) increases [13].

Our interpretation of these data concerns the role and interactions of the various SSS and SBE forms. The *shx* mutation specifically affects a Type I SSS [3,15]. The Type I forms are primer-independent in vitro, suggesting they may be able to synthesize new molecules, whereas the primer-dependent Type II SSS may instead elongate existing α-D-glucans. If Type I SSS is primarily responsible for the initiation of the biosynthesis

of new molecules, *shx* may have fewer than normal amylopectin molecules, contributing to the reduced A-granule dimensions observed [16]. This specific effect on A-granule dimensions remains to be elucidated, but may relate to the modulation observed for SSS during the development of barley grains [3]. The unaffected Type II activity would, together with SBE, respectively elongate and branch the molecules laid down by the two Type I forms unaffected in *shx*.

Observations on the *C. reinhardtii st*-3 mutant are consistent with distinct roles for Type I and Type II SSS, since the lessened SSS II cannot be complemented by Type I activity (S. Ball, personal communication). This is not necessarily inconsistent with proposals for distinct initial glucosylation events [44] which could reflect the production of endogenous primer for the Type I SSS. In *st*-3, branching may in fact be normal, in that the branch points are formed (as indicated by the presence of synthesis of dp 2–6 "stubs"), although these short B-chains appear then not to be elongated due to lack of SSS Type II [13].

We have earlier observed [16] that amylose content is increased only from 26 to 28% (w/w) in *shx*, although the ratio of GBSS activity, which is unaffected, relative to SSS activity is higher than normal. It appears, therefore, that there may be limits to the total proportion of starch which may be synthesized as amylose. Indeed, the "high amylose" mutants investigated to date affect amylopectin and not the amylose complement. The phenotype of the maize *ae*, for example, consists of the amylose as produced by GBSS and an increased content of intermediate and long chains in the amylopectin [7,9,10,12] which have amylosic properties. This change has been brought about by the lack of SBE form IIa [8,45]. Similar observations have been made for SBE mutations in rice [46] which manifest specific changes in branching pattern. The various SBE forms have been shown by in vitro assays to preferentially transfer chains in different length ranges [47], and by sequence comparisons to comprise two distinct groups [48].

Taken together, our data and those of others indicate that the structure of amylopectin depends on the distinct roles of the SSS and SBE forms present, rather than on overall relative ratios of chain-elongation versus chain-transfer activity. This view must to some extent be tempered by the paucity of *sss* mutants informing the true in vivo enzymology of the diverse SSS forms in various plants. A final resolution of the relationship between amylopectin structure and the function of the various forms of SSS, GBSS, and SBE will await pure enzyme preparations and defined substrates for in vitro biosynthetic reactions and the combinatorial expression of the corresponding genes in transgenic organisms.

References

[1] A.M. Smith and F. Martin, in D. Grierson (Ed.), *Plant Biotechnology*, Vol. 3, Blackie, London, 1993, pp 1–54.
[2] J. Preiss, *Oxford Survey of Plant Molecular and Cellular Biology*, 7 (1992) 59–114.
[3] J. Tyynelä and A.H. Schulman *Physiol. Plant.*, 89 (1993) 835–841.
[4] F.D. Macdonald and J. Preiss, *Plant Physiol.*, 78 (1985) 849–852.
[5] W. Banks, C.T. Greenwood, and J.T. Walker, *Staerke*, 22 (1970) 149–180.
[6] Y.-J. Wang, P. White, L. Pollak, and J. Jane, *Cereal Chem.*, 70 (1993) 171–179.

[7] H. Fuwa, D.V. Glover, K. Myaura, N. Inouchi, Y. Konishi, and Y. Sugimoto, *Starch / Staerke*, 39 (1987) 295–298.
[8] C.D. Boyer and J. Preiss, *Plant Physiol.*, 67 (1981) 1141–1145.
[9] Y. Ikawa, D.V. Glover, Y. Sugimoto, and H. Fuwa, *Starch / Staerke*, 33 (1981) 9–13.
[10] N. Inouchi, D.V. Glover, T. Takaya, and H. Fuwa, *Starch / Staerke*, 35 (1983) 371–376.
[11] N. Inouchi, D.V. Glover, Y. Sugimoto, and H. Fuwa, *Starch / Staerke*, 36 (1984) 8–12.
[12] C.D. Boyer and K.-C. Liu, *Starch / Staerke*, 37 (1985) 73–79.
[13] T. Fontaine, C. D'Hulst, M.-L. Maddelein, F. Routier, T.M. Pépin, A. Decq, J.-M. Wieruszeski, B. Delrue, N. Van Den Koornhuyse, J.-P. Bossu, B. Fournet, and S. Ball, *J. Biol. Chem.*, 268 (1993) 16223–16230.
[14] H. Ahokas, *Hereditas*, 114 (1991) 281–284.
[15] A.H. Schulman and H. Ahokas, *Physiol. Plant.*, 78 (1990) 583–589.
[16] A.H. Schulman, R.F. Tester, H. Ahokas, and W.R. Morrison, *J. Cereal Sci.*, 19 (1994) 49–55.
[17] J. Tyynelä, M. Stitt, A. Lönneborg, S. Smeekens, and A.H. Schulman, *Physiol. Plant.*, 93 (1995) 77–84.
[18] C.F. Jenner, K. Siwek, and J.S. Hawker, *Aust. J. Plant Physiol.*, 20 (1993) 329–335.
[19] P.L. Keeling, P.J. Bacon, and D.C. Holt, *Planta*, 191 (1993) 342–348.
[20] A.M.L. McDonald and J.R. Stark, *J. Inst. Brew., London*, 94 (1988) 125–132.
[21] Y. Takeda, S. Hizukuri, and B.O. Juliano, *Carbohydr. Res.*, 148 (1986) 299–308.
[22] S. Lansky, M. Kooi, and T.J. Schoch, *J. Am. Chem. Soc.*, 71 (1949) 4066–4075.
[23] Y. Takeda, S. Hizukuri, and B.O. Juliano, *Carbohydr. Res.*, 168 (1987) 79–88.
[24] B.L. Larson, K.A. Gilles, and R. Jennes, *Anal. Chem.*, 25 (1953) 802–804.
[25] A. Suzuki, S. Hizukuri, and Y. Takeda, *Cereal Chem.*, 58 (1981) 286–290.
[26] S. Hizukuri and S. Osaki, *Carbohydr. Res.*, 63 (1978) 261–264.
[27] S. Hizukuri, Y. Takeda, M. Yasuda, and A. Suzuki, *Carbohydr. Res.*, 94 (1981) 205–213.
[28] M. Somogyi, *J. Biol. Chem.*, 195 (1952) 19–23.
[29] N. Nelson, *J. Biol. Chem.*, 153 (1944) 375–380.
[30] S. Hizukuri, S. Tabata, and Z. Nikuni, *Starch / Staerke*, 10 (1970) 338–343.
[31] M. Dubois K.A. Gilles, J.K. Hamilton, P.A. Rebers, and F. Smith, *Anal. Chem.*, 28 (1956) 350–356.
[32] Y. Takeda and S. Hizukuri, *Biochim. Biophys. Acta*, 185 (1969) 469–471.
[33] S. Hizukuri, K. Shirasaka, and B.O. Juliano, *Starch / Staerke*, 35 (1983) 348–350.
[34] S. Hizukuri and T. Takagi, *Carbohydr. Res.*, 134 (1984) 1–10.
[35] T. Takagi and S. Hizukuri, *J. Biochem. (Tokyo)*, 95 (1984) 1459–1467.
[36] W.R. Morrison, D.C. Scott, and J. Karkalas, *Starch / Staerke*, 38 (1986) 374–379.
[37] R.F. Tester and W.R. Morrison, *Cereal Chem.*, 69 (1992) 654–658.
[38] K. Shibanuma, Y. Takeda, and S. Hizukuri, *Carbohydr. Polym.*, 25 (1994) 111–116.
[39] Y. Takeda, N. Maruta, S. Hizukuri, and B.O. Juliano, *Carbohydr. Res.*, 187 (1989) 287–294.
[40] S. Hizukuri, Y. Takeda, N. Murata, and B.O. Juliano, *Carbohydr. Res.*, 189 (1989) 227–235.
[41] Y. Takeda, T. Shitaozono, and S. Hizukuri, *Starch / Staerke*, 40 (1988) 51–54.
[42] A. Suzuki, M. Kaneyama, K. Shibanuma, Y. Takeda, J. Abe, and S. Hizukuri, *Cereal Chem.*, 69 (1992) 309–315.
[43] A.W. MacGregor and J.E. Morgan, *Cereal Chem.*, 61 (1984) 222–228.
[44] F.J. Ardila and J.S. Tandecarz, *Plant Physiol.*, 99 (1992) 1342–1347.
[45] P.L. Dang and C.D. Boyer, *Biochem. Genet.*, 27 (1989) 521–532.
[46] K. Mizuno, T. Kawasaki, H. Shimada, H. Satoh, E. Kobayashi, S. Okumura, Y. Arai, and T. Baba, *J. Biol. Chem.*, 268 (1993) 19084–19091.
[47] Y. Takeda, H.-P. Guan, and J. Preiss, *Carbohydr. Res.*, 240 (1993) 253–263.
[48] R.A. Burton, J.D. Bewley, A.M. Smith, M.K. Bhattacharyya, H. Tatge, S. Ring, V. Bull, W.D.O. Hamilton, and C. Martin, *Plant J.*, 7 (1995) 3–15.