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Phosphorylation of C6- and C3-positions of glucosyl residues in starch is catalysed by distinct dikinases

Gerhard Ritte[a,*], Matthias Heydenreich[b], Sebastian Mahlow[a], Sophie Haebel[c], Oliver Kötting[d], Martin Steup[a]

[a] *Plant Physiology, Institute of Biochemistry and Biology, University of Potsdam, Karl-Liebknecht-Strasse 24-25, Building 20, 14476 Potsdam-Golm, Germany*
[b] *Analytical Chemistry, Institute of Chemistry, University of Potsdam, Karl-Liebknecht-Strasse 24-25 Building 25, 14476 Potsdam-Golm, Germany*
[c] *Interdisciplinary Center of Mass Spectrometry of Biopolymers, University of Potsdam, Karl-Liebknecht-Strasse 24-25, Building 20, 14476 Potsdam-Golm, Germany*
[d] *Institute of Plant Sciences, ETH Zürich, Universitätstrasse 2, CH-8092 Zürich, Switzerland*

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Abstract **Glucan, water dikinase (GWD) and phosphoglucan, water dikinase (PWD) are required for normal starch metabolism. We analysed starch phosphorylation in Arabidopsis wild-type plants and mutants lacking either GWD or PWD using ^{31}P NMR. Phosphorylation at both C6- and C3-positions of glucose moieties in starch was drastically decreased in GWD-deficient mutants. In starch from PWD-deficient plants C3-bound phosphate was reduced to levels close to the detection limit. The latter result contrasts with previous reports according to which GWD phosphorylates both C6- and C3-positions. In these studies, phosphorylation had been analysed by HPLC of acid-hydrolysed glucans. We now show that maltose-6-phosphate, a product of incomplete starch hydrolysis, co-eluted with glucose-3-phosphate under the chromatographic conditions applied. Re-examination of the specificity of the dikinases using an improved method demonstrates that C6- and C3-phosphorylation is selectively catalysed by GWD and PWD, respectively.**

Keywords: Starch phosphorylation; GWD; PWD; ^{31}P NMR

1. Introduction

Starch of most plant sources contains a low percentage of glucosyl residues that are monoesterified with phosphate. Usually, less than 0.5% of the glucosyl residues are phosphorylated but the precise value varies depending upon plant organ and species. In potato tuber starch (the most intensively studied material) approximately 70–80% and 20–30% of the phosphate is bound to the C6- and C3-positions, respectively [1–3].

The analysis of starch phosphorylation poses technical difficulties. All sensitive methods to analyse the different phosphate monoesters rely on acid hydrolysis of starch, which ideally gives rise to glucose, glucose-6-phosphate (Glc6P), and glucose-3-phosphate (Glc3P). Glc6P can be analysed by an enzymatic test, but for Glc3P this is not possible. Blennow et al. separated glucose, Glc6P, and Glc3P (derived from controlled acid hydrolysis of starch) by high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) [4]. In principle, this method allows for the analysis of C3-linked phosphate in starch. However, the C3-bound phosphate is rather acid labile and it is, therefore, difficult (or impossible) to hydrolyse all the glucosidic linkages without loosing some Glc3P. Blennow et al. estimated that approximately 20% of the Glc3P was lost during hydrolysis (2 h in 0.7 N HCl at 100 °C). Putative conversion products of Glc3P with retention times very similar to that of authentic Glc3P were also reported as products of acid hydrolysis of starch [4]. Therefore, this method does not allow for a precise quantification of C3-bound phosphate. Alternatively, ^{31}P NMR can be used to study starch phosphorylation [2,5]. Acid hydrolysis of starch is not required for ^{31}P NMR analysis, but a major drawback of this method is its low sensitivity. Thus, NMR is not applicable if only small amounts of starch are available.

Covalently bound phosphate in starch affects its physicochemical properties and thereby its usability for different industrial processes [6]. However, the importance of starch phosphorylation for plant metabolism and the related enzymology were uncovered only recently. Suppression of a starch-associated protein (preliminarily designated as R1) in potato led to a decrease in the phosphate content and degradability of starch [7]. Subsequently, we showed that R1 is a glucan, water dikinase (GWD) which phophorylates starch-like glucans in an ATP-dependent reaction [8]

$$\alpha\text{-glucan} + \mathrm{ATP} + \mathrm{H_2O} \rightarrow \alpha\text{-glucan-P} + \mathrm{AMP} + \mathrm{P_i}$$

Following in vitro phosphorylation the glucans were subjected to acid hydrolysis and the products were analysed by HPAEC-PAD. Based on these studies it was concluded that GWD phosphorylates both C6- and C3-positions with a preference for C6 [8,9]. Subsequently, a second starch phosphorylating enzyme was identified in Arabidopsis [9,10]. Interestingly, its activity is strictly dependent on starch being pre-phosphorylated by GWD and it was, therefore, named phosphoglucan, water dikinase (PWD). Based on the same analytical approach, purified recombinant PWD (also designated as GWD3) was reported to phosphorylate predominantly or exclusively the C3-position of the glucose moieties [9,10]. However, no or a rather moderate reduction in the

*Corresponding author. Fax: +49 331 9772512.
E-mail address: ritte@rz.uni-potsdam.de (G. Ritte).

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putative C3-phosphate esters was observed in starch of PWD-deficient Arabidopsis plants relative to wild-type [9,10].

Arabidopsis mutants lacking either GWD or PWD exhibit significantly increased leaf starch contents with the phenotype of the GWD-deficient plants being more severe [9–11]. Thus, both proteins are required for normal starch turnover in Arabidopsis. Presently, the effect(s) of glucan phosphorylation on starch degradability cannot be explained on a molecular level and it is also not known if there are any selective functions of C6- and C3-bound phosphates. The elucidation of metabolic functions of the different phosphate esters is also affected by the methodological difficulties discussed above.

Here, we analysed phosphate in starch extracted from leaves of Arabidopsis wild-type and mutant plants using ^{31}P NMR. The content of C3-bound phosphate was close to the limit of detection in plants lacking PWD. Re-investigation of the specificity of GWD and PWD using an improved methodology clearly shows that the phosphorylation of the C6- and the C3-positions of glucosyl residues in starch is catalysed by distinct enzymes.

2. Materials and methods

2.1. Plant material and growing conditions

Arabidopsis wild-type plants (ecotype Columbia) and *pwd* mutants [9] were cultivated in a growth cabinet (12 h light/12 h dark, 20/16 °C, 70% RH, and approximately 150 μmol quanta m^{-2} s^{-1}). After four weeks, plants were transferred to 14 h light/10 h dark, 20 °C, and approximately 275 μmol quanta m^{-2} s^{-1} for four days until harvest. The mutants *sex1*-3 and SALK_077211 (designated as *sex1*-8) were grown under 14 h light/10 h dark, 22/17 °C, 70% RH, and approximately 100 μmol quanta m^{-2} s^{-1}.

2.2. Chemicals

[β^{33}P]ATP (10 mCi/ml, 800 Ci/mmol) and [^{33}P]phosphoric acid (54 mCi/mL, carrier free) were purchased from Hartmann Analytic (Braunschweig, Germany). Glc3P was synthesized as described elsewhere [8].

2.3. Preparation of α-limit dextrins and NMR analysis

Leaves were harvested at the end of the light period and frozen at −80 °C until use. Particulate starch was prepared from Arabidopsis leaves as described [9] and was converted into α-limit dextrins according to Lim and Seib [2] with some modifications. Starch (225 mg) was suspended in 1.5 ml 6 mM NaCl, 2 mM $CaCl_2$. One hundred units of α-amylase from *Bacillus* spec. (A-6380, Sigma) dissolved in 40 μl 6 mM NaCl, 2 mM $CaCl_2$ was added and the suspension was incubated for 10 min at 95 °C with shaking to gelatinize and simultaneously liquify the starch. The suspension was cooled, additional enzyme was added (200 U), and the digestion was continued for 2 h at 65 °C with shaking. The reaction was terminated by heating at 100 °C for 15 min. Insoluble material was removed by centrifugation and the supernatant was concentrated to a volume of approximately 0.3 ml using a speed vac. Samples were then adjusted to 10 mM citrate–NaOH (pH 4.2), 20% (v/v) D_2O, 5 mM EDTA, 0.12% (w/v) sodium azide in a final volume of 0.75 ml.

^{31}P NMR spectra were recorded using a BRUKER AVANCE 300 spectrometer at 121.45 MHz. 26624 transients were accumulated. Chemical shifts were referenced to external 85% H_3PO_4. All experiments were acquired and processed with standard BRUKER software.

2.4. Preparation of maltose-6-phosphate (Mal6P)

Phosphorylated oligo-saccharides were prepared from potato amylopectin [12]. Maltose-6-phosphate (Mal6P) was obtained from α-limit phosphodextrins by acid hydrolysis as described [1] with the following modifications. Approximately 50 mg phosphodextrins were hydrolysed in 1.3 ml of 0.35 M H_2SO_4 for 2.5 h at 99 °C. Following neutralisation with $Ba(OH)_2$ the sample was applied to anion exchange chromatography using a smaller column (V = 1.3 ml) and a flow rate of 0.65 ml/min. In the eluate fractions (1.3 ml each) reducing ends were determined [13] and selected fractions were further analysed by HPAEC-PAD, spectrophotometric determination of Glc6P, and MALDI-MS. Maltose-phosphate containing fractions were combined and concentrated under vacuum. NMR analysis revealed that the phosphorylated position was the C6 at the non-reducing glucosyl residue (data not shown) which is in agreement with the previous study [1]. As revealed by MALDI-MS small amounts of maltotriose-phosphate were also present in the sample. By using this procedure, approximately 1.3 μmol Mal6P were obtained with a purity of $\geqslant$ 80%.

2.5. HPAEC-PAD analyses

HPAEC-PAD analysis was performed using a Dionex DX600 HPLC-System. Two different methods were applied:

Method A (PA-100 column, flow rate 1 ml/min): Samples ($\leqslant$80 μl) were injected after a 10 min equilibration step using 5 mM Na–acetate dissolved in 100 mM NaOH. Elution was performed using a linear Na–acetate gradient (0–30 min: 5–500 mM) in 100 mM NaOH. The column was washed for 10 min with 500 mM Na–acetate in 100 mM NaOH.

Method B (PA-200 column, flow rate 0.5 ml/min): Following equilibration (as above) up to 20 μl sample was injected. The eluent contained 100 mM NaOH and the following Na–acetate concentrations: 0–11 min: 5–150 mM (linear gradient), 11–25 min: 150 mM, 25–30 min: 150–500 mM (linear gradient), 30–40 min: 500 mM.

2.6. In vitro phosphorylation of starch

Recombinant potato GWD [8] and recombinant Arabidopsis PWD [9] were purified and the radioactive starch phosphorylation assays were performed as described [9]. However, 1 mM dithioerythritol was included in the assays and following termination of the reaction the granules were washed with 2% (w/v) SDS, 2 mM ATP (see [9]) and finally two times with water. The starch granules (5 mg) were then dried under vacuum, mixed with 150 μl of 0.7 N HCl and incubated at 95 °C with shaking. After 2 h 30 μl were removed and neutralised with 0.7 N NaOH. The remaining suspension was hydrolysed for additional 2 h and then neutralised. Prior to HPAEC-PAD analysis samples were centrifuged through 10 kDa membranes [9].

2.7. Nanoelectrospray quadrupole time of flight (NanoESI Q-TOF) mass spectrometry

MS/MS spectra were recorded using a API QSTAR pulsar I (Applied Biosystems/MDS Sciex, Toronto, Canada) hybrid mass spectrometer equipped with a nanoelectrospray ion source. The ion of interest was selected in the Q1 quadrupole. Fragments were generated in the collision cell by collision with Argon and analysed in the TOF mass analyser.

3. Results

Starch phosphorylation was analysed in Arabidopsis wild-type plants and the previously described mutants *pwd* [9,10] and the GWD-deficient line *sex1*-3 [11]. In addition the mutant SALK_077211 (designated as *sex1*-8) was also analysed. In this mutant the *GWD* gene is disrupted by a T-DNA insertion. In homozygous *sex1*-8 plants no GWD protein could be detected using Western Blot (data not shown), growth was reduced, and the leaf starch content at the end of a 14 h light period was approximately 5 times higher than that of the wild-type plants. This phenotype closely resembles those of the previously described GWD-deficient mutants [11].

α-Limit dextrins prepared from leaf starch were analysed using ^{31}P NMR. Four peaks were obtained in the wild-type sample (Fig. 1, WT). The dominant signal 3 at δ 0.96 ppm represents phosphate bound to the C6-position as indicated by the ^{1}H-coupled ^{31}P spectrum. Signal 3 splits into a triplet as a result of coupling of the phosphorus nucleus with the two

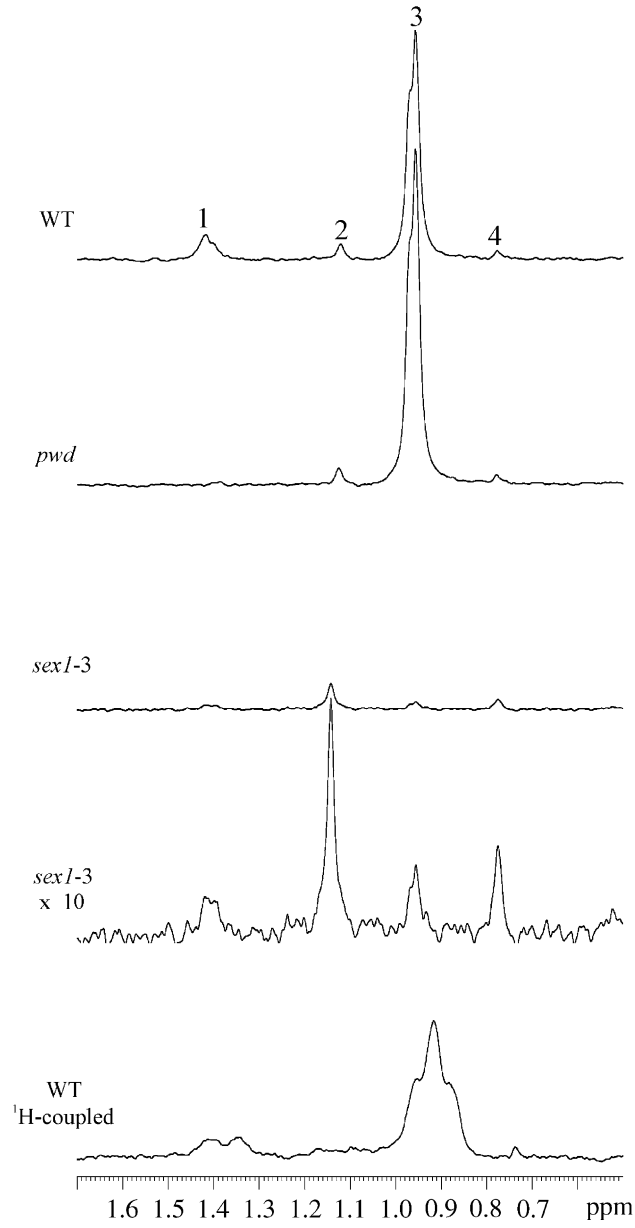

Fig. 1. ^{31}P NMR analysis of α-limit dextrins prepared from Arabidopsis leaf starch. Chemical shifts were referenced to H_3PO_4 as external standard. Peak areas are proportional to the relative amount of phosphorus. In the ^{1}H-coupled spectrum of the WT sample (lower panel) peak 1 splits up into a doublet and peak 3 splits up into a triplet. This is caused by coupling of the phosphorus nuclei with one or two neighbouring proton(s), respectively. Therefore, peak 1 is assigned as C3-bound phosphate and peak 3 is attributed to C6-bound phosphate.

neighbouring protons bound to carbon atom 6 (Fig. 1, WT ^{1}H-coupled). The shape of peak 3 (Fig. 1, WT) indicates that it is composed of more than one species. This is because phosphodextrins of different chain length are produced by α-amylase and the resonance is also affected by the specific position of the phosphorylated glucose residue within the glucan chain. The second largest signal 1 at δ 1.42 ppm (Fig. 1, WT) splits into a doublet in the ^{1}H-coupled ^{31}P spectrum (Fig. 1, WT ^{1}H-coupled). Thus, this peak represents phosphorus bound to a carbon to which a single H is attached. Signal 1 is, therefore, assigned as C3-bound phosphate. In α-limit dextrins prepared from *pwd* plants C3-phosphate esters were reduced to levels close to the limit of detection (Fig. 1 and Table 1). Thus, PWD appears to be essential for phosphorylation at the C3-position. The content of C6-bound phosphate was significantly increased in the *pwd* sample relative to wild-type as revealed by integration of the peak areas (Table 1). This was confirmed by acid hydrolysis of these starch samples and quantification of Glc6P and glucose. In the samples prepared from wild-type and *pwd* plants, the Glc6P/glucose ratios (in nmol/μmol) were 0.65 and 0.96, respectively. Transgenic Arabidopsis plants in which PWD was reduced by means of RNAi [9] also showed an approximately 30% increase in starch-bound Glc6P residues. In starch prepared from *sex1*-3 and *sex1*-8 plants both C6- and C3-phosphorylation were drastically reduced, but still detectable (Table 1). The minor signal 4 most likely represents inorganic phosphate since it gives rise to a single peak only in the coupled spectrum (Fig. 1). The identity of signal 2 which is present in all samples with similar intensity is unknown. It was reported that approximately 1% of the starch bound phosphate in potato tubers might be linked to the C2-position of glucosyl residues [1]. Alternatively, signal 2 could be derived from a non-carbohydrate starch component such as phosphoprotein or phospholipid.

Table 1
Relative amounts of phosphorylated compounds in starch of wild-type and mutants

	Relative peak area			
	Peak 1 C3-P	Peak 2 unknown	Peak 3 C6-P	Peak 4 P_i
Wild-type	13.2	5.5	100	2.6
pwd	<1[a]	5.6	150.5	1.9
sex1-3	2.4	9.2	3.1	2.2
sex1-8	2.5	9.3	2.8	1.5

The peak areas of the spectra shown in Fig. 1 were quantified relative to the peak area of C6-bound phosphate in the wild-type sample. In addition a ^{31}P NMR spectrum of α-limit dextrins prepared from *sex1*-8 plants was also analysed.
[a]Inspection of the spectrum at 50-fold magnification indicates that traces of C3-bound phosphate might be present.

It should be noted that the analysis of starch phosphorylation by ^{31}P NMR is not suited to process a large number of samples. To generate acceptable signal to noise ratios in the ^{31}P NMR spectra each sample was analysed for 24 h. The amount of starch needed for NMR analysis also limits the application of these technique if organs with rather low starch content (e.g. leaves) are to be examined.

The apparent lack of C3-bound phosphate in *pwd* plants contrasts with previous reports on this mutant [9,10] and also with the presumed C3-phosphorylation catalysed by GWD [8,9]. In the previous studies starch was hydrolysed with HCl, the products were subjected to HPAEC-PAD and substances were identified based on their retention time as compared to authentic Glc6P and Glc3P. A possible explanation for the conflicting results would be co-elution of another substance with Glc3P in the HPAEC separation, thus leading to false conclusions. To test this possibility hydrolysed starch (potato tuber starch was used because of its higher phosphate content) was subjected to HPAEC-PAD (method A, Section 2), the peak ascribed to Glc3P was collected and subsequently analysed using ESI-Q-TOF-MS. Compounds were detected with masses equivalent to glucose-phosphate and maltose-

phosphate, respectively. The compounds were further characterised by fragmentation analysis using MS–MS. Authentic Glc6P and Glc3P served as standards. The glucose-phosphate was identified as Glc3P, whereas the fragments obtained from the putative maltose-phosphate indicated that the phosphate was bound to the C6- rather than to the C3-position. For a more detailed analysis maltose-6-phosphate (Mal6P) was prepared as a reference substance [1]. The fragments obtained by MS–MS analysis of authentic Mal6P matched those of the compound that co-eluted with Glc3P (data not shown).

Because of the co-elution of Mal6P with Glc3P under the chromatographic conditions applied in previous studies [8,9] we had to re-examine the specificity of the purified dikinases with respect to the phosphorylation of the C6- and/or C3-positions of glucosyl residues using a modified procedure. ^{31}P NMR could not be applied because of its low sensitivity. Therefore, we modified the HPAEC to allow for a separation of the glucose-phosphates and Mal6P. Using a different column and a modified gradient (method B, Section 2) Glc6P, Glc3P and Mal6P could clearly be separated (Fig. 2). The sugar phosphates are detected with different sensitivity. The sensitivity for Glc3P is approximately 5 and 8 times lower than that for Glc6P and Mal6P, respectively.

To analyse the specificity of GWD, starch granules were phosphorylated in vitro using the recombinant potato enzyme and [β^{33}P]ATP. Following acid hydrolysis the samples were mixed with internal standards and subjected to HPAEC (method B) and radioactivity in the collected fractions was determined (Fig. 3). The results clearly show that GWD phosphorylates exclusively the C6-position of the glucosyl residues. After 2 h of hydrolysis 15% of the incorporated radioactivity eluted with Mal6P. After 4 h of hydrolysis this proportion decreased to 3% and essentially all radioactivity eluted with Glc6P. No label co-eluted with Glc3P.

The specificity of recombinant PWD of Arabidopsis was also re-examined. Essentially, all of the incorporated radioactivity eluted with Glc3P and hydrolysis derived conversion products of Glc3P (Supplementary Figs. 1 and 2). Labeling of Glc6P or Mal6P was insignificant (Supplementary Fig. 2). Thus, it can be concluded that PWD selectively phoshorylates the C3-position.

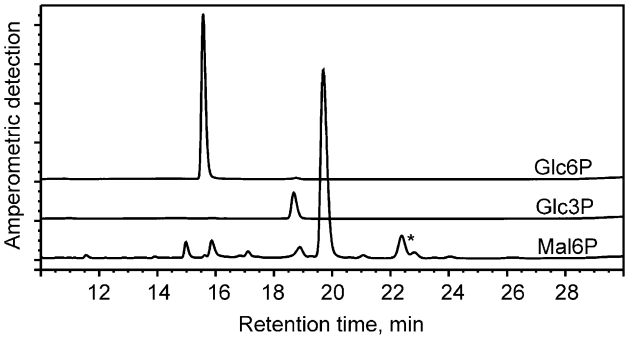

Fig. 2. Separation of glucose-phosphates and Mal6P by HPAEC-PAD using method B. Glc6P, Glc3P (1 nmol each) and Mal6P (approximately 1 nmol) were injected. The Mal6P preparation contains small amounts of contaminants. The peak labeled with an asterisk comprises a maltotriose-phosphate.

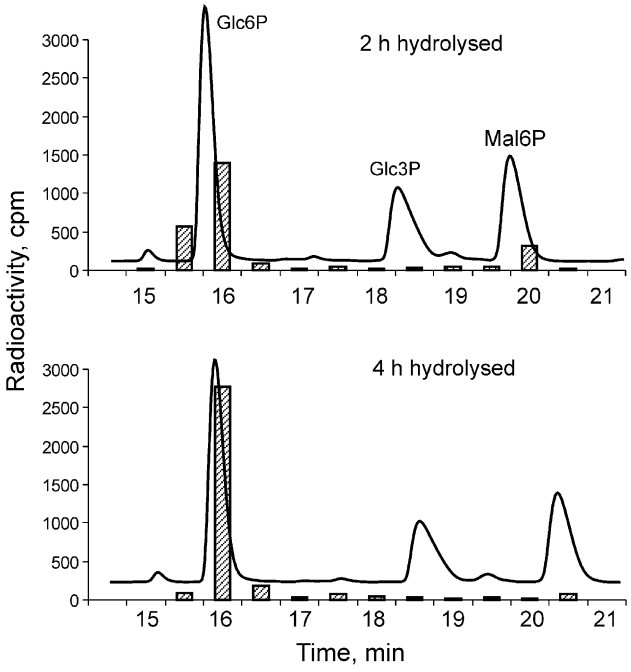

Fig. 3. GWD phosphorylates the C6-position of glucosyl-residues. Starch granules (5 mg) isolated from *sex1*-3 mutant leaves were phosphorylated in vitro by recombinant potato GWD (3.2 μg) with 4.5 μM ATP containing 1.6 μCi ^{33}P-ATP for 40 min. Total phosphate incorporation was 0.22 nmol. Following removal of the free label, granules were subjected to acid hydrolysis for 2 or 4 h, respectively. Aliquots of the hydrolysates were mixed with Glc6P (4 nmol), Glc3P (8 nmol) and Mal6P (2 nmol) as internal standards and subjected to HPAEC-PAD (method B). Radioactivity in the collected fractions was counted. Glucose the most prominent compound in the samples eluted after 3 min. For clarity only the sections in which labeled compounds elute are shown.

4. Discussion

In the present communication we show that GWD exclusively phosphorylates the C6-position of glucosyl residues in starch, whereas PWD catalyses the phosphotransfer to C3, but not to C6. These conclusions were reached by re-investigation of the specificity of the starch phosphorylating enzymes in vitro using an improved methodology and by ^{31}P NMR analysis of starch extracted from Arabidopsis wild-type plants and mutants defective in either GWD or PWD. It has been shown previously that PWD activity strictly depends on a preceding action of GWD [9,10] and it is now evident that PWD relies on C6-bound phosphate. Consequently, not only C6- but also C3-bound phosphate was drastically reduced in GWD-deficient plants. ^{31}P NMR analysis also showed that minute amounts of Glc6P-residues are still present in starch of GWD null mutants. These tiny levels were below the detection limit using enzymatic test or HPAEC-PAD analysis [11]. In addition to GWD and PWD the existence of another putative starch phosphorylating dikinase (designated as AtGWD2 [10]) was predicted from the analysis of the Arabidopsis genome [11]. AtGWD2 is not yet fully characterised but the remaining starch phosphorylation at C6 in *sex1*-3 and *sex1*-8 plants might be due to this isoenzyme.

In the last few years there has been significant progress in the identification of enzymes involved in synthesis or degradation of starch [14–16]. However, less is known on the regulation of

these processes. The phenotypes of the GWD-antisense potato plants [7] and the Arabidopsis mutants *sex1* and *pwd* [11,9,10] suggest that glucan phosphorylation is involved in the coordination of synthesis and degradation of starch. Phosphorylation occurs during both synthesis [17,18] and degradation [18] of starch and in *Chlamydomonas reinhardtii* phosphate is incorporated at positions C6 and C3 of the glucosyl residues under both conditions. The latter result was obtained by HPAEC-PAD analysis (method B) of hydrolysed starch that had been extracted from ^{33}Pi fed algae (data not shown). Presently, it is not clear why normal turnover of starch depends on two dikinases that act in series and phosphorylate different positions of glucosyl residues. Phosphate esters affect the packing of the amylopectin helices and modeling studies indicate that C3-bound phosphate likely exerts a greater effect on helix packing than phosphate linked to the C6-position [6]. Furthermore, phosphorylation of starch increases its hydrophilicity [6]. It is possible that phosphate esters locally open up the structure of the semicrystalline starch particle, thereby accelerating the attack of degrading enzymes. Alternatively or additionally, the activity of certain starch metabolising enzymes could rely on specific interactions with phosphate linked to the C6- or C3-position, respectively. Since it is now known that distinct enzymes phosphorylate the two different positions, the investigation of particular roles of C6- and C3-bound phosphate is possible. The specific manipulation of C6- and C3-phosphorylation in transgenic crop plants by increasing or decreasing the activities of GWD or PWD also opens the possibility to produce starch with novel functionality.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2006.07.085.

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