OPEN ACCESS
Scholars International Journal of Biochemistry
Abbreviated Key Title: Sch Int J Biochem
ISSN 2616-8650 (Print) |ISSN 2617-3476 (Online)
Scholars Middle East Publishers, Dubai, United Arab Emirates
Journal homepage: https://scholarsmepub.com/sijb/

Original Research Article

Identify the Specificity of Interaction between the Arabidopsis Starch Synthase 4 and the Plastidial Starch Phosphorylase using a Homologous Protein-Animal Rabbit Muscle Phosphorylase a

Hadeel Mohammed Qasim[1], Julia Compart[1], Slawomir Orzechowski[2], Joerg Fettke[1] and Shadha Abduljaleel AL-Rawi[1*]

[1]Biopolymer Analytics, University of Potsdam, 14476 Potsdam-Golm, Germany
[2]Department of Biochemistry, Warsaw University of Life Sciences, -SGGW, 02-776 Warsaw, Poland

DOI: 10.36348/sijb.2019.v02i12.003 | **Received:** 30.11.2019 | **Accepted:** 07.12.2019 | **Published:** 28.12.2019

***Corresponding author:** Shadha Abduljaleel AL-Rawi

Abstract

The starch synthase 4 (SS4) is a key enzyme for initiation of starch granules and regulation of the starch granule number in chloroplasts of higher plants. These enzymes transfer glucosyl residue from ADPglucose to the non-reducing end of a preexisting glucan chain. The presence of a coiled-coil motive in the N-terminus of Arabidopsis SS4 has been involved in mediating some of the protein-protein interactions. Thus, it was also shown that *At*SS4 directly interacts with the plastidial phosphorylase (AtPHS1). However, phosphorylase enzymes are widespread in animals, microorganism, and plants. So far, it was unclear if the observed protein-protein interaction is specific for plant origin phosphorylase enzymes. Therefore, we tested whether or not an animal type phosphorylase, the rabbit muscle phosphorylase a (Pho a), also interacts with AtSS4. Our results show that the protein-protein interaction of AtPHS1 and AtSS4 is specific and cannot archived by Pho a. Furthermore, also a functional interaction between AtSS4 and the Pho a was not detected.

Keywords: Arabidopsis thaliana, plastidial phosphorylase, starch synthase, rabbit muscle phosphorylase, protein interaction, protein crosslinking.

Copyright @ 2019: This is an open-access article distributed under the terms of the Creative Commons Attribution license which permits unrestricted use, distribution, and reproduction in any medium for non-commercial use (NonCommercial, or CC-BY-NC) provided the original author and source are credited.

INTRODUCTION

Carbohydrates are one of the main types of biomolecules, playing a vital role in the living organism. The two most abundant storage carbohydrates are starch and glycogen. Starch is a polysaccharide that consists of two polymers amylose and amylopectin in both the glucosyl residues are linked by α 1-4 glycosidic bond with a lesser frequency of α 1-6 linkages in amylose, so it is defined as a major linear glucan, and significant amount in amylopectin [1, 2] Like amylopectin glycogen is a polymer that is exclusively formed by glucose units that are linked by α 1-4 and α 1-6 glycosidic linkage. These linkages formed during the starch and glycogen biosynthesis by the activity of starch synthases (SSs) with branching (BE) and debranching (DBE) enzymes and glycogen synthase (GS) with glycogen branching enzyme (GBE), respectively. However, the formed α 1-6 branches are randomly distributed in glycogen but were ordered in amylopectin.

In addition to the chemical similarities of both glucans, the mode of action of the metabolizing enzymes is also clearly related [3]. For instance, starch phosphorylase (AtPHS1 in Arabidopsis or Pho1 in other plant species) and glycogen phosphorylase (Pho a) have a similar function, as both of them catalysis the reversible exchange of glucosyl units between glucose-1-phosphate (G-1-P) and the non-reducing ends of α 1-4 glucans. In higher plants, PHS1 play a dynamic role in starch metabolism, thus in degradation and synthesis [4-6]. Pho1 plays a crucial role in starch synthesis in plastids of non-photosynthetic storage tissue of higher plants, as the loss of Pho1 caused smaller starch granules and accumulation of a modified amylopectin structure. Recently, more biochemical and genetic studies revealed that PHS1/Pho1 play a key role in the initiation and formation of storage starch. Barley Pho1 was reported to initiation α 1-4 glucan synthesis from G-1-P alone, based on experiments where preparations of enzyme and substrate missing the potential acceptor glucans [7]. In rice, Pho1 can work in starch priming by elongating short glucan chains [6] which can be generated during starch degradation by the activity of α-amylase (AMY3), β-amylase (BAM) and isoamylase

(ISO3) [8]. Besides the plastidial phosphorylase to elongating the short glucan chains, there are several other plastidial enzymes that can act *e.g.* disproportion enzyme (DPE1) which has the ability to release a portion from the non-reducing end of one glucan chain and connect it to another non-reducing end thereby forming short and long glucan chains [9, 10]. Also starch synthases (SSI, SSII and SSIII) are involved in the elongation of α-glucan chains of amylopectin [11]. Recently, a new hypothesis proposes protein-protein or multi-protein complex machinery for starch metabolism, for instance, starch synthase 4 (AtSS4) which possess a coiled-coil motive in its structure led to the interaction with other starch metabolizing proteins [12]. However, AtSS4 is required for granule initiation [13]. Interaction of AtSS4 and AtPHS1 was shown [14]. Thus, we tested the specificity of the interaction of starch synthase 4 with the Arabidopsis plastidial phosphorylase by using a non-plant, phosphorylase- the rabbit muscle phosphorylase a (Pho a).

MATERIAL AND METHODS

Starch granules were isolated from Arabidopsis leaves as described by [15]. Glycogen phosphorylase was purchased from Sigma Aldrich product No.-232-878-8 and the isoamylase (200U/ml) from Megazyme. 8-aminopyrene-1, 3-6trisulfonic acid (ATPS) and sodium Cyanoborhydrid were purchased from Sigma Aldrich.

Cloning, expression, and purification of recombinant AtSS4 and AtPHS1proteins

AtSS4 and AtPHS1 were cloned as described elsewhere [16, 2]. The proteins were expressed without transit peptide using pET23b vector (Novagen) and the *E. coli* strain BL21 (DE3). For expression, cells were grown in 800ml (37C°) Luria-Bertani medium containing 100µg/ml ampicillin. Expression (overnight at 20C°) was induced by addition of isopropyl thio-β-galactoside (IPTG 1mM final concentration) at OD 600nm values between 0.6 and 0.8. Harvesting of the cells and purification of His-tagged protein were performed as described [16]**.** Purified protein fractions were concentrated via ultrafiltration (50kDa; Amicon Ultra; Millipore) in 50mM HEPES/NaOH, pH 7.5, 1mM EDTA, 2mM DTE and 10% (w/v) glycerol. Aliquots were frozen in liquid nitrogen and stored at -80°C until use. Protein concentration was estimated using Bradford assay (SIGMA, Taufkirchen, Germany).

SDS-PAGE, Western blotting, and immune detection

Fractions were analyzed by SDS-PAGE and the proteins were transferred to a membrane see [17]. The membrane was analyzed by antibodies specific against AtSS4 and AtPHS1, respectively.

Protein-Protein Interaction Studies

The interaction between AtPHS1 and AtSS4 by using a cross linker

10mg of a cross linker DTSSP was dissolved in 495µl of dry DMF solvent. The cross linker was used in a 20–fold excess compared to the proteins. The final cross linker concentration was 0.5mM and optimal pH range was from 7 to 9. 40µg of the recombinant plastidial phosphorylase and 200µg of crude extract isolated from Col-0 were incubated with cross linker DTSSP for 5min at 21°C. Quench of any unreacted DTSSP was performed by using 25M of Tris, pH 7.4 and allowed to react for 10-15min, 21°C. Following purification of the recombinant AtPHS1 by using Ni-NTA agarose column and washing 6 times with 1mL of 50mM Imidazole the proteins were eluted by 6 mL 150mM Imidazole. After purification all fractions were transferred to ultrafiltration units (Amicon®, 10kDa Millipore, and Billerica, MA, United States) and centrifuged for 12min at 4°C, 13000rpm. Finally, all fractions were analyzed by SDS–PAGE (7.5%) and incubated with anti–SS4 antibody. In addition, the same procedure was performed but without recombinant plastidial phosphorylase.

Confirm the interaction between AtPHS1 and AtSS4 by using α-amylase

40µg of the AtPHS1 and 200µg protein crude extract were incubated in the presence of 20 units α-amylase for 10min at 37°C. After the incubation the purification were done by using the Ni-NTA agarose as described above.

The interaction between AtPHS1 and both enzymes AtSS1 and AtSS3

250µg crude extract proteins isolated from Col-0 were incubated with 40µg of AtPHS1 and the recombinant protein was isolated by Ni-NTA agarose as described above. The eluting fractions were loaded on native gel. As a control 250µg crude extract were directly loaded on native gel. After electrophoreses the gels were incubated in buffer that contained 50mM Tricin/KOH pH.8, 25mM K-acetate, 5mM DTE, 2mM EDTA, 0.02% (w/v) bovine serum albumin, and 1mM ADPglucose overnight at room temperature and stained with iodine.

The interaction between AtSS4 and Pho a using the cross linker DTSSP, 40µg of AtSS4 and 40µg of Pho a were incubated in presence of the cross linker DTSSP and the recombinant AtSS4 was purified and analyzed by native-PAGE as described above. For washing and elution 9mL 50mM and 9mL 150mM Imidazol were used.

Native-PAGE and phosphorylase activity staining

Native-PAGE followed by phosphorylases activity staining was performed as described by [14].

Glucan synthesizing assay and glucan preparation

0.5mg of native starch granules were resuspended in incubation buffer [50mM HEPES/KOH pH 7.4 ,1mM DTE, 10mM G-1-P] with 10µg of glycogen Pho a or 5µg of starch phosphorylase AtPHS1 alone or in presence of 5µg AtSS4. The samples were incubated for 60min at 30C° under continuous agitation and were centrifuged at 1500g for 2min. The supernatant was then discarded and the starch pellets were resuspended in 10mM Na- acetate buffer, pH 5.5, 2mM DTE, 7 units isoamylase (final volume 100µl over nights at 37°C) under continuous agitation. The samples were centrifuged for 10min at 1500g; the supernatant was heated at 95°C for 5min and were filtrated (10kDa), lyophilized, labeled, and analyzed using CE-LIF as described in [17].

CE-LIF analysis

Chain length distribution analysis of the samples were performed by measuring the ATPS-labeled α 1-4 glucans using a PA-800 (Beckman coulter) equipped with a laser-induced fluorescence detector (LIF). The total length of the N-CHO-coated capillary (i.d., 50µm) was 50cm. The running buffer was a mixture of 25mM Li-acetate (pH 4.75) and 0.4 (w/v) polyethylene oxide. The separation was performed for 25min at 30kV and 25°C.

RESULTS

Conformation of the direct interaction of AtPHS1 with AtSS4

As published in [14] a direct protein-protein interaction was observed for the plant derived plastidial phosphorylase and the starch synthase 4. We therefore test if this interaction is observed also under our experimental conditions. In *E. coli* heterologous expressed AtPHS1, that has a C-terminal 6x His-tag, was incubated with crude extract of Arabidopsis leaves harvested in the middle of the light period. Following incubation for five minutes at 21°C the recombinant AtPHS1 was purified by a Ni-NTA agarose column. The proteins bound to the column were eluted and analyzed using SDS-PAGE, western blotting, and immune detection using a specific antibody against AtSS4. As shown in Figure (1, A) the interaction between AtSS4 and AtPHS1 was confirmed. As control an identical experiment was performed but the recombinant AtPHS1 was omitted. In this control experiment no signal for AtSS4 was observed, excluding any unspecific co-purification of the enzyme by this procedure (Figure 1, A). The co-purification is specific for AtSS4, as no interaction with AtSS1 and AtSS3 was observed (Fig. 1, B).

No protein interaction was observed for the rabbit muscle phosphorylase a with AtSS4

We then test if the rabbit muscle phosphorylase a also interacts with AtSS4. Therefore, we incubated purified rabbit muscle phosphorylase a directly with purified recombinant AtSS4 C-terminal fused to a 6x His-tag. Following incubation for five minutes at 21°C the His-tagged AtSS4 was purified using a Ni-NTA agarose column. The eluted protein fraction was tested for the presence of the rabbit phosphorylase a using a native-PAGE and staining for phosphorylase activity. No phosphorylase was co-purified with AtSS4 (Fig. 1, C). As maybe the interaction of the rabbit muscle Pho a and AtSS4 is much weaker and thus during the purification procedure the phosphorylase is not co-purified, we included the cross-linking by DTSSP. Therefore, we test again first the procedure with both plant derived enzymes and observed again a clear interaction also using the crosslinking protocol (Fig. 1, C). However, using the same procedure for Pho a and AtSS4, we did not observe any phosphorylase activity (Fig. 1, C). Thus, even a very weak protein-protein interaction between these two proteins is very unlikely.

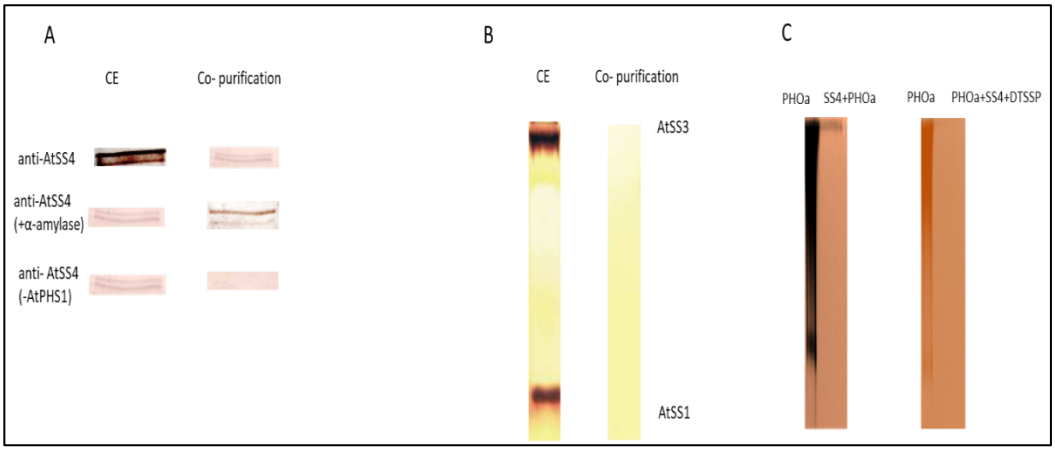


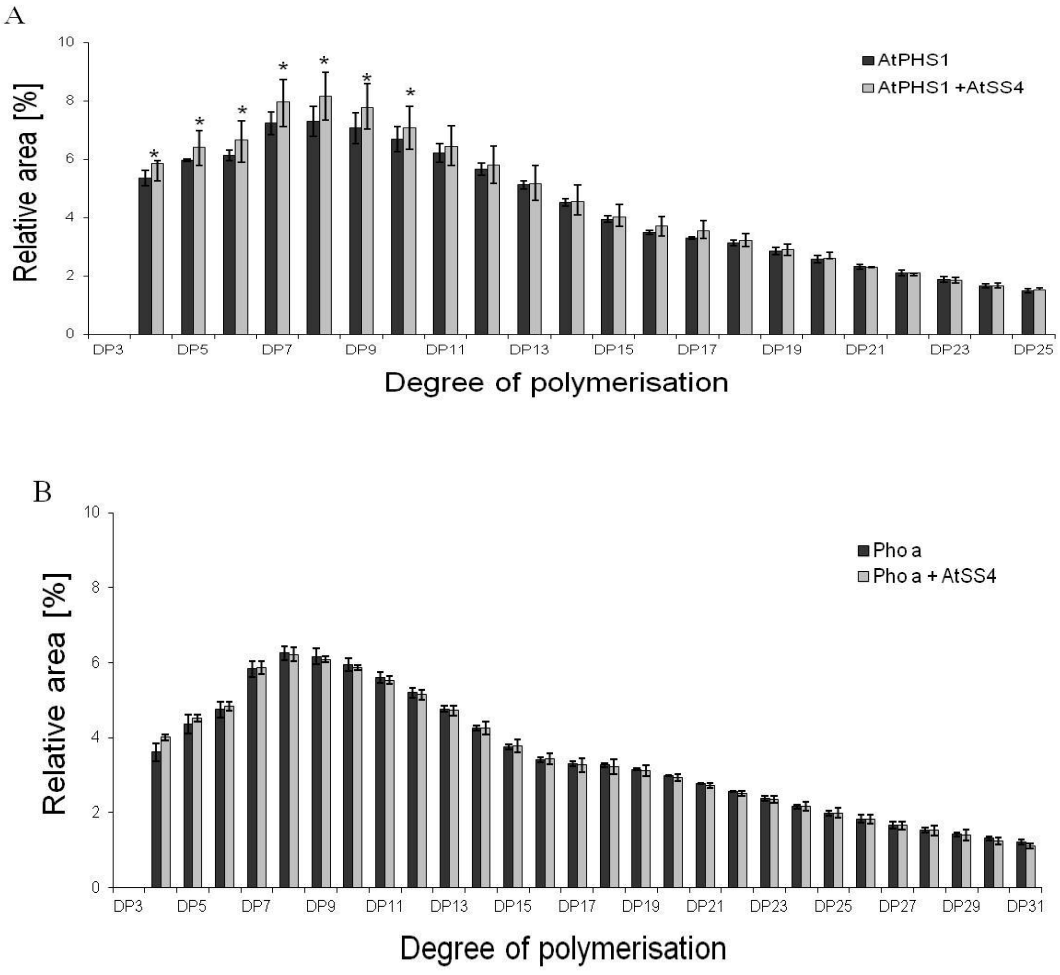
Fig-1(A)

Fig 1(A): The interaction of starch synthase 4 (AtSS4) with the recombinant plastidial phosphorylase (AtPHS1). 40µg C-terminal 6x His tagged recombinant plastidial phosphorylase protein from *Arabidopsis thaliana* expressed in *E.coli* was incubated with 200µg protein crude extract (CE) isolated from Col-0 for 5min at 21°C. Following purification of the recombinant plastidial phosphorylase using Ni-NTA agarose

column, all fractions were analyzed by an antibody specific for AtSS4. In addition, in order to rule out the possibility of the correlation between AtSS4 and AtPHS1 the recombinant plastidial phosphorylase and the protein crude extract (CE) isolated from Col-0 were incubated with 20 units α-amylase for 10min at 37°C. As a control the recombinant plastidial phosphorylase was omitted (AtSS4-AtPHS1). (**B**): 200µg of the crude extract with recombinant plastidial phosphorylase were incubated then purified with the column, the fractions were loaded on native-PAGE and incubated with buffer containing 50mM Tricin/KOH pH 8, 25mM K-acetate, 5mM DTE, 2mM EDTA, 0.02% (w/v) bovin serum albumin and 1mM ADPglucose over night at room temperature and stained with iodine. Thus no co-purification of plastidial phosphorylase with AtSS1 or AtSS3 was observed. (**C**): 40µg of the starch synthase 4 and 40µg of non-plant phosphorylase a from rabbit muscle was incubated with the cross linker DTSSP for 5min at 21°C. Following purification using Ni-NTA agarose column for the recombinant AtSS4, washing, and elution with Imidazole (50 and 150mM) all fractions were loaded on native-PAGE and after electrophoreses incubated for phosphorylase activity staining. Thus, no co-purification of phosphorylase a and starch synthase 4 was observed.

Simultaneous incubation of AtPHS1 and AtSS4 revealed alterations of the glucans accessible by isoamylase at the surface of starch

We did not observe any direct protein-protein interaction for phosphorylase a and AtSS4, thus we analyzed whether or not the proteins functional interact during glucan synthesis. Therefore, we incubated AtSS4 with AtPHS1 or Pho a in presence of glucose-1-phosphate and starch as substrate. As control AtSS4 was omitted. The glucan products were analyzed after 1h incubation in synthesizing direction. Following debranching of the starch surface by isoamylase treatment, the glucans were analyzed by CE-LIF. We observed slightly different glucan distribution patterns for both phosphorylase. Thus incubation with the plant derived AtPHS1 resulted in elongation of short glucan chains compared to the incubation with the animal phosphorylase a. However, for the plant derived phosphorylase a small increase of shorter glucan chains were detected in the presence of AtSS4 that was significant, even very weak. However, the resulting patterns for the animal phosphorylase showed no significant alterations in presence or absence of AtSS4 (Fig. 2). Thus, no evidence for a functional interaction was observed.



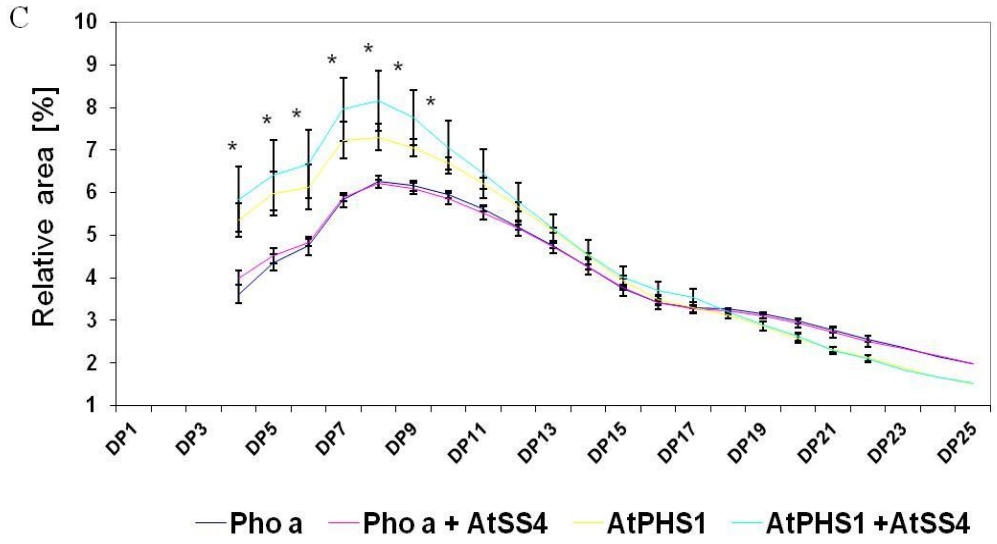


Fig-2

Fig 2: Chain length distribution of Arabidopsis starch formed by the interaction of AtPHS1 or Pho a with AtSS4. (A, B): 5mg Arabidopsis starch were incubated with 10µg Pho a or 5µg AtPHS1 alone as a control or with 5µg AtSS4.The pellets were debranched with isoamylase, glucans were labeled with APTS, and then separated and detected using CE-LIF. Values are the means ± SD (n=3). C: Deference plots to compare the effect of AtSS4 on the action of Pho a and AtPHS1. Values are the means ± SD (n=3) *, $P \leq 0,5$ according to Student's t-test.

DISCUSSION

Recently more biochemical and genetic studies reveal the role of AtSS4. Thus, it was shown that AtSS4 is involved in the process of starch initiation by formation of the priming molecule, but also appeared to be required for other activities during starch synthesis. However, the precise role has not yet been clarified [18]. All classes of SS contain a highly conserved C-terminal end that included the glucosyl transferase catalytic domains (GT5) and (GT1). The N- terminal regions differ in size and amino acid sequence [19]. AtSS4 differs from the other classes in containing two long coiled-coil domains, extended from the (GT5) domain [20, 21].This region is highly conserved and led to the suggestion that it is important for protein-protein interactions [12, 22]. Recently, more evidences revealed that interaction of SS4 with the other proteins is important for granule initiation for instance in Arabidopsis, thus three proteins were identified, belonging to the protein targeting to starch (PTST) family, PTST1, PTST2, and PTST3 [23, 24]. Other results showed that AtSS4 interact with the protein fibrillin1a and 1b (FBN 1a and FBN 1b), respectively which are mainly located in the plastoglobules (PGs) [25]. Furthermore, a protein named PII1 (protein involved in starch initiation) was identified as an interacting protein of AtSS4. Also the *pii1* mutant revealed a reduced number of starch granules compared to the wild type [26]. It was also reported, that AtSS4 interacts with the plastidial starch phosphorylase (AtPHS1) [14]. In the present study we tested whether or not this reported interaction is specific for plant phosphorylase or can this interaction also be observed by non-plant phosphorylases. Therefore, we included the rabbit muscle phosphorylase a. Both proteins share the same biochemical reaction that catalyses the reversible reaction of α 1-4 glucan and inorganic phosphate to glucose-1-phosphate and a glucan reduced in length by one glucosyl unit from the non-reducing end. We analyzed the direct protein-protein interaction and showed that AtSS4 and AtPHS1 interact, as reported by [14]. However, no interaction of AtSS4 and Pho a was observed (Fig.1, C) .As we know all phosphorylases consist of dimers or tetramers of identical subunits. Further, their kinetic and structural properties are also similar but, they differ in their regulation mechanisms which depend on the source of these enzymes [4]. Thus, the presence of specialized interaction between AtSS4 and AtPHS1 can be explained by the origin of these enzymes.

Furthermore, we analyzed if a functional interaction can be detected. Therefore, we incubated both phosphorylases with starch and glucose 1-phosphate (synthesizing reaction), and analyzed the formed product by CE-LIF in presence and absence of AtSS4. However, the significant elongation of short glucan chains in the presence of AtSS4 with AtPHS1 indicates a functional interaction between both proteins (Fig. 2, A). By contrast, no significant alterations were observed in the presence of AtSS4 with Pho a, thus a functional interaction can be excluded (Fig 2, B). However, missing of the functional interaction between AtSS4 and Pho a and the clear detection with AtPHS1 uncover a potential function for AtSS4 with AtPHS1 during starch synthesis. Several studies revealed that

starch phosphorylase has a certain effect during starch synthesis rather than during degradation in higher plants. While, it was shown that the increasing of starch phosphorylase activity coincides with starch accumulation in wheat, developing rice cereal endosperms, and [5, 6, 27].

Our study showed that Arabidopsis plastidial phosphorylase (AtPHS1) specifically interacts with AtSS4 that cannot be replaced by Pho a. However, so far it is unclear whether or not other plant derived phosphorylases can replace the Arabidopsis enzyme and if all animal phosphorylases don't show an interaction with the AtSS4. However, our data support the idea that during starch synthesis the plastidial phosphorylase and the starch synthase 4 interact.

REFERENCES

1. Ball, S.G., Morell, M.K. (2003). From bacterial glycogen to starch: Understanding the biogenesis of the plant starch granule. Annu Rev Plant Biol, 54: 207–233.
2. Fettke, J., Fernie, A. R., Steup, M. (2012). Transitory starch and its degradation in higher plant cells. In: Essential Reviews in Experimental Biol. Vol. 5: The Synthesis and Breakdown of Starch. I.J. Tetlow, ed. The Society for Experimental Biology, London, 309–372.
3. Ball, S., Colleoni, C., Cenci, U., Raj, J. N., Tirtiaux, C. (2011). The evolution of glycogen and starch metabolism in eukaryotes gives molecular clues to understand the establishment of plastid endosymbiosis. Journal of Experimental Botany, *62*, 1775-180
4. Dauville, D., Chochois, V., Steup, M., Haebel, S., Eckermann, N., Ritte, G., Ral, J. P., Colleoni, C., Hicks, G., Wattebled, F., Deschamps, P., d'Hulst, C., Liénard, L., Cournac, L., Putaux, J. L., Dupeyre, D., Ball, S.G. (2006). Plastidial phosphorylase is required for normal starch synthesis in Chlamydomonas reinhardtii. Plant J. 48: 274–285.
5. Schupp, N., Ziegler, P. (2004). The Relation of Starch Phosphorylases to Starch Metabolism in Wheat. Plant Cell Physiol, 45: 1471–1484.
6. Satoh, H., Shibahara, K., Tokunaga, T., Nishi, A., Tasaki, M., Hwang, S. K., Thomas, W., Nanae, O., Naoko, K., Mayumi, F., Yuko, Y., Aya Sato, H., Utsumi, Y., Ohdan, T., Nakamura, Y. (2008). Mutation of the Plastidial a-Glucan Phosphorylase Gene in Rice Affects the Synthesis and Structure of Starch in the Endosperm. The Plant Cell. 20: 1833–1849.
7. Cuesta-Seijo, J. A., Ruzanski, C., Krucewicz, K., Meier, S., Hägglund, P., Svensson, B., Palcic, M. M. (2017). Functional and structural characterization of plastidic starch phosphorylase during barley endosperm development. PLOS One 12, 1–25.
8. Seung, D., Thalmann, M., Sparla, F., Abou Hachem, M., Lee, S. K., Issakidis-Bourguet, E. Svensson, B., Zeeman, S. C., Santelia, D. (2013). arabidopsis thaliana AMY3 is a unique redox-regulated chloroplastic α- amylase. Journal of Chemistry, 288, 33620-33633.
9. Critchley, J. H., Zeeman, S. C., Takaha, T., Smith, A. M., Smith, S. M. (2001). A critical role for disproportionating enzyme in starch breakdown is revealed by a knock- out mutation in Arabidopsis. Plant Journal, 26, 89-100.
10. Kartal, Ö, Mahlow, S., Skupin, A., Ebenhöh, O. (2011). Carbohzdrateßactive enyzmes exemplifz entropic principles in metabolism. Molecular Szstms Biology 7, 542.
11. Brust, H., Orzechowski, S., Fettke, J., Steup, M. (2013). Starch synthesizing reaction and path: in vitro and in vivo studies. Journal of Applied Glycoscience, 60, 3-20.
12. D'Hulst, C., Mérida, Á. (2010). The priming of storage glucan synthesis from bacteria to plants: current knowledge and new developments. New Phytologist, 188, 13-21.
13. Roldán, I., Wattebled, F., Mercedes Lucas, M., Delvallé, D., Planchot,V., Jiménez, S., Pérez, R., Ball, S., D'Hulst, C., Mérida, A. (2007). The phenotype of soluble starch synthase IV defective mutants of arabidopsis thaliana suggests a novel function of elongation enzymes in the control of starch granule formation. Plant Journal, 49,492-504.
14. Malinova, I., Qasim, H. M., Brust, H., Fettke, J. (2018). Parameters of starch granule genesis in chloroplasts of Arabidopsis thaliana. Frontiers in Plant Science, 9, 1-7.
15. Malinova, I., Mahlow, S., Alseekh, S., Orawetz, T., Fernie, A. R., Baumann, O., Steup, M., Fettke, J. (2014). Double knock-out mutants of Arabidopsis thaliana grown under normal conditions reveal that the plastidial phosphorylase isozyme (PHS1) participates in transitory starch metabolism. Plant Physiol. 164, 907–921.
16. Brust, H., Lehmann, T., D'Hulst, C., Fettke, J. (2014). Analysis of the Functional Interaction of Arabidopsis Starch Synthase and Branching Enzyme Isoforms. Reveals that the Cooperative Action of SSI and BEs Results in Glucans with Polymodal Chain Length DistributionSimilartoAmylopectin.PLoSONE9(7):e 102364.
17. Malinova, I., Mahlow, S., Alseekh, S., Orawetz, T., Fernie, A. R., Baumann, O., Steup, M., Fettke, J. (2014). Double knock-out mutants of Arabidopsis thaliana grown under normal conditions reveal that the plastidial phosphorylase isozyme (PHS1) participates in transitory starch metabolism. Plant Physiol, 164, 907–921.
18. Seung, D., Lu, K-J., Stettler, M., Streb, S., Zeeman, S.C. (2016). Degradation of glucan primers in the absence of Starch Synthase 4 disrupts starch

granule initiation in Arabidopsis. Journal of Biological Chemistry, 291, 20718–20728.
19. Leterrier, M., Holappa, L. D., Broglie, K. E., Beckler, D. M. (2008). Cloning, characterisation and comparative analysis of a starch synthase IV gene in wheat: functional and evolutionary implications. BMC Plant Biol. 8, 98–119.
20. Rose,A., Manikantan,S., Schraegle,S., Maloy,M., S tahlberg,E., Meier,I. (2004). Genome- wide identification of Arabidopsis coiled- coil proteins and establishment of the ARABI- COIL database. Plant Physiol, 134, 927– 939.
21. Raynaud, S., Ragel, P., Rojas, T., Mérida, Á. (2016). The N-terminal part of Arabidopsis thaliana Starch Synthase 4 determines the localization and activity of the enzyme. Journal of Biological Chemistry, 291, 10759–10771.
22. Szydlowski, N., Rage, l. P., Raynaud, S., Mercedes, M. L., Roldán, I., Montero, M., Muñoz, F. J., Ovecka, M., Bahaji, A., Planchot, V., Pozueta-Romero, J., D'Hulst, C., Mérida, Á. (2009). Starch granule initiation in Arabidopsis requires the presence of either class IV or class III starch synthases. Plant Cell, 21, 2443– 2457.
23. Seung, D., Soyk, S., Coiro, M., Maier, B.A., Eicke, S., Zeeman, S.C. (2015). Protein targeting to starch is required for localising granule-bound starch synthase to starch granules and for normal amylose synthesis in Arabidopsis. PLOS Biology 13, e1002080.
24. Seung, D., Boude,t J., Monroe, J.D., Schreier, T.B., David, L. C., Abt, M, Lu, K-J., Zanella, M., Zeeman, S. C. (2017). Homologs of protein targeting to starch control starch granule initiation in Arabidopsis leaves. Plant Cell, 29, 1657–1677.
25. Gámez-Arjona, F. M., Raynaud, S., Ragel, P., Mérida, Á. (2014). Starch synthase 4 is located in the thylakoid membrane and interacts with plastoglobule-associated proteins in Arabidopsis. Plant Journal, *80*(2): 305–316.
26. Vandromme, C., Spriet, C., Dauvillee, D., Courseaux, A., Putaux, J.L., Wychowski, A., Krzewinski, F., Facon, M., D'Hulst, C., Wattebled, F. (2019). PII1: a protein involved in starch initiation that determines granule number and size in Arabidopsis chloroplast. New Phytologist 221:356–370.
27. Yu, Y., Mu, H. H., Mu-Forster, C., Wasserman, B. P., George, M. C. (2001). Identification of the maize amyloplast stromal 112-kD protein as a plastidic starch phosphorylase. *Plant Physiol.* 125: 351-359.