LIKE SEX4 1 Acts as a β-amylase-binding Scaffold on Starch Granules During Starch Degradation

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Review timeline:

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	1st Decision:	March 22, 2019 *revision requested*
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REPORT: (The report shows the major requests for revision and author responses. Minor comments for revision and miscellaneous correspondence are not included. The original format may not be reflected in this compilation, but the reviewer comments and author responses are not edited, except to correct minor typographical or spelling errors that could be a source of ambiguity.)

TPC2019-00089-RA 1st Editorial decision – *revision requested* **March 22, 2019**

All three reviewers commented on the quality and the advancement of the field represented by your work. Reviewer #3 raises the point that it is not possible to demonstrate the absence of something, i.e., the absence of catalytic activity in the case of LSF1. To address this point, please carefully revise the manuscript to make specific statements as to which activities are undetectable (avoid "absent") under the conditions used in your study. Reviewer #3 is further concerned that the native PAGE protocol used in your work may not be optimal for detecting all enzyme activities of interest. This comment can be addressed by providing a more comprehensive description of the procedure in the Methods section of the manuscript and by running out the samples on an alternate native PAGE system that does not lead to strongly alkaline conditions during the separation procedure.

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[Reviewer comments shown below along with author responses]

TPC2019-00089-RAR1 1st Revision received **May 17, 2019**

Reviewer comments and **author responses**:

Reviewer #1:

This well-written and very well-documented article shows a facilitating role for the LSF1 protein whose function was unknown or poorly understood until now and whose knock-out mutation leads to an excess starch phenotype. This work, mainly focused on the biochemistry of the protein, is considerable (as evidenced by the length of the Methods) and rigorous, including four different approaches to demonstrate that LSF1 interacts with beta-amylases BAM1 and BAM3, thus facilitating their action on starch. Truncated forms but also point mutants of LSF1 were generated, which made it possible to understand how the CBM (carbohydrate binding-module) domain plays a central role in the action of the protein, and that the DSP domain is secondary. This is a new piece of the complex puzzle of starch degradation that has been posed. It is a major step forward, and congratulations to the authors.

Points in favor: The subject is important. The regulation of starch degradation is still poorly understood. The facilitator role of a protein whose loss of function leads to a starch excess phenotype is elucidated. The strategy used is perfectly adapted and the results are sound.

No points detracting.

Point 1. Frankly, the article is great. Maybe in the Discussion we would like to have more ideas about the follow-up to this work, namely how LSF1 is involved in starch degradation, for example does LSF1 influence the turnover of BAM1 or BAM3? or is LSF1's facilitating action used to modulate the rate of starch degradation?

RESPONSE: We highly appreciate the reviewer's comments on the manuscript. We agree with the suggestions for follow up work and have incorporated these ideas into the final paragraph of the Discussion (line 545-567).

Reviewer #2:

This manuscript describes the in-depth study of LSF1, a protein involved in starch degradation, as shown in previous work. Here the authors show that LSF1, conversely to SEX4 homolog protein, has no phospho-glucan phosphatase activity. Indeed, amylopectin is phosphorylated, triggering starch degradation in Arabidopsis leaves at night. Therefore, the involvement of LSF1 protein in starch degradation is not due to its DSP (Dual Specificity Phosphatase) domain. However, mutations of tryptophan residues in the CBM48 domain render LSF1 unable to bind to starch and to complement the *lsf1* mutant. The authors also found that LSF1 physically interacts with BAM1 and BAM3. These latter two are beta-amylases involved in leaf starch degradation at night in Arabidopsis. Thus, the starch excess phenotype of the *lsf1* mutant is not a consequence of the lack of a putative phosphatase activity, but is probably due to the absence of LSF1 protein binding at the starch granule surface. The results presented in this manuscript are convincing and the work of good quality. The results are interesting since they provide new insights about the way starch is degraded in Arabidopsis leaves, suggesting that LSF1 could acts as a beta-amylase scaffold required for optimal control of starch degradation.

I don't have major comments about this work, only few and rather minor requests/remarks:

Point 1. Fig 2: What is the meaning of lines "1" and "2" in Fig 2B and 2D? Is it 2 biological replicates with 3 individual plants each? This is not clear. Same remark applies to Supplemental Fig. 2C

RESPONSE: We have now clarified in the Figure captions that these are two different homozygous lines from independent transformation events (line 979-980, line 10681069).

Point 2. Fig 2 (and elsewhere in the manuscript): "equal area leaf basis"? What is the rationale of such standardization? Why not protein or chlorophyll content?

RESPONSE: Equal leaf area basis was chosen for practical reasons. The different genotypes did not vary in leaf thickness or rosette morphology, and thus we harvested leaf discs (hence equal leaf area) for making the protein extracts.

Point 3. Line 217: Why such PTM would only exist for the modified protein and not for the native one? It seems to be a large PTM, looking at the shift in the gel (20-30kDa). This was not observed with the PDZ and the DSP/CBM deleted forms of LSF1 (Suppl Fig. 2A). Any explanation?

RESPONSE: We do not yet know the reason for the two different bands in the Flag-HA tagged transgenic lines. We agree that it is rather peculiar that the double band is not seen with the native protein. However, these immunoblots (Figure 2B showing the transgenic protein, and Figure 1D from Comparot-Moss et al. (2010)) were performed with two different antibodies: anti-Flag in the case of Figure 2B, and anti-LSF1 in the latter. It is possible that the LSF1 antibody used by the former study cannot detect the upper band (possibly due to the PTM).

However, we also mention the possibility that the upper band could represent the protein with its chloroplast transit peptide uncleaved (line 214-215), indicating that the Flag-HA tagged transgenic proteins may be imported to the chloroplast less efficiently. The predicted chloroplast transit peptide for LSF1 is 61 amino acids long and 6.78 kDa (according to the TargetP 1.1 Server). While the reason for the double band for the Flag-HA tagged proteins is unknown, we argue that the transgenic proteins are fully functional. The tagged LSF1 WT protein expressed under its native promoter in the *lsf1* mutant background complements the starch-excess phenotype of *lsf1*.

Reviewer #3:

Starch is a water-insoluble particle usually composed of two types of polyglucans, amylopectin and amylose. Amylopectin is the major polysaccharide type of starch. Amylose - the minor glucan type - may even be undetectable. In both types, glucosyl moieties are essentially linked by only two bonds, α-1,4 linkages (more than 90% of the interglucose linkages, forming chains) and, to a minor extent, α-1,6 linkages, which lead to branches.

During biosynthesis of amylopectin, chains are elongated at the non-reducing end by a glucosyl transfer mediated by the various starch synthase isoforms or the plastidial phosphorylase isozyme(s). Elongation of amylopectin is, therefore, strictly unidirectional. Recently, the knowledge of starch metabolism, especially that of transitory starch, has significantly increased. Assimilatory starch is mainly degraded by hydrolytic attack through various β-amylase isoforms, which liberates maltose from the non-reducing ends of glucan chains and, therefore, is also unidirectional.

Obviously, the (de)polymerizing enzyme activities mentioned before do not allow the selective starch structure to be constructed. Debranching of amylopectin also occurs during starch biosynthesis and, presumably, is required to obtain obtain clustering of branchings. Furthermore, a small protein family (PTST) has been discovered that does not catalyze any chemical reaction but possesses both a carbohydrate binding and a protein interacting domain; the function of this protein family appears to be to target enzymes in the vicinity of the assimilatory starch. Finally, two amylopectin-phosphorylating and two dephosphorylating enzymes have been identified that appear to affect the starch structure.

The present manuscript focuses on the function of one of the putative dephosphorylating enzymes, Like Starch Excess Four 1 (LSF1). The authors provide some evidence that despite sequence homology, LSF1 lacks catalytic activity as a phosphatase but rather interacts with other proteins, mainly β-amylase 1 or 3, and uses its carbohydrate-binding and protein interacting domain for placing either of both hydrolases in close vicinity of the starch granule. These interesting results may be correct but are not yet fully convincing. Certainly it is quite difficult to clearly state that - despite sequence homology - a protein lacks any catalytic activity.

To give a few examples:

Point 1. In the manuscript, the LSF1 protein was mostly used as a tagged polypeptide, which appears to be expressed in Arabidopsis as a double band (Fig. 1B), although the endogenous LSF1 protein seems to exist as a single form. The authors suggest that the doublet is due to a covalent modification (Line 212-216). No evidence for this assumption is presented. Furthermore, the ratio between the two bands varies depending upon the respective construct. Some constructs seem not to accumulate.

RESPONSE: See comment above.

Point 2. The manuscript contains both clear statements that LSF1 lacks any catalytic activity in vitro (Fig. 1; Line 949) and more cautious versions (Line 390-391; Line 441-443).

RESPONSE: We agree with the reviewer that we cannot completely exclude the possibility that LSF1 might have phosphatase activity that is undetectable due to incorrect assay conditions or substrate. However, we can conclude that any phosphatase activity at the DSP domain is not responsible for the starch-excess phenotype of the *lsf1* knock-out mutant. We made adjustments in the text accordingly (see line 1000).

Point 3. It is difficult to see why sometimes the conserved cysteine is named catalytical (Line 164) or conserved active (Line 195), although any catalytic activity is rejected.

RESPONSE: We thank the reviewer for pointing out the inconsistency. We now refer to the residue as the conserved cysteine of the Dual Specificity Phosphatase (DSP) domain throughout the manuscript.

Point 4. The native PAGE used appears to be the Laemmli System without SDS. This discontinuous system separates at a strongly alkaline pH value (it is known that during separation, the pH value of the buffer of the separation gel increases significantly). Some starch-related proteins are inactivated by this alkaline pH value. Unfortunately, no information is given on how long proteins were separated under these conditions.

RESPONSE: We have added this information to the Methods section (line 808-809). We thank the reviewer for pointing out that the alkaline running conditions could be a reason why BAM3 activity was not detected. We added this possibility to the manuscript (line 357-363).

TPC2019-00089-RAR1 2nd Editorial decision – *acceptance pending* June 4, 2019

We are pleased to inform you that your paper entitled "LIKE SEX4 1 acts as a β-amylase-binding scaffold at the starch granule during starch degradation" has been accepted for publication in The Plant Cell, pending a final minor editorial review by journal staff. At this stage, your manuscript will be evaluated by a Science Editor with respect to scientific content presentation, compliance with journal policies, and presentation for a broad

readership.

Final acceptance from Science Editor	**June 26, 2019**