



Research article

Silencing of α -amylase *StAmy23* in potato tuber leads to delayed sprouting

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ABSTRACT

Potato tuber dormancy is critical for the postharvest quality. The supply of carbohydrates is considered as one of the important factors controlling the rate of potato tuber sprouting. Starch is the major carbohydrate reserve in potato tuber, but very little is known about the specific starch degrading enzymes responsible for controlling tuber dormancy and sprouting. In this study, we demonstrate that an α -amylase gene *StAmy23* is involved in starch breakdown and regulation of tuber dormancy. Silencing of *StAmy23* delayed tuber sprouting by one to two weeks compared with the control. This phenotype is accompanied by reduced levels of reducing sugars and elevated levels of malto-oligosaccharides in tuber cortex and pith tissue below the bud eye of *StAmy23*-deficient potato tubers. Changes in soluble sugars is accompanied by a slight variation of phytyloglycogen structure and starch granule size. Our results suggest that *StAmy23* may stimulate sprouting by hydrolyzing soluble phytyloglycogen to ensure supply of sugars during tuber dormancy.

1. Introduction

Potato (*Solanum tuberosum* L.) is the most important non-grain crop worldwide, which requires a continuous supply throughout the year and long-term storage after harvest. At harvest and for a finite period thereafter, potato tubers are physiologically dormant and will not sprout. However, the break of dormancy and sprouting adversely affect the nutritional and processing features of potatoes during the post-harvest storage (Coleman, 1987; Sonnewald, 2001; Börnke et al., 2007; Sonnewald and Sonnewald, 2014). Therefore, tuber dormancy control is very important for the potato industry, however, the underlying mechanism with respect to the release and maintenance of tuber dormancy is still unclear. Understanding the regulation mechanism of tuber sprouting is essential to regulate post-harvest tuber dormancy.

The length of the dormancy period depends on the genetic background of the cultivar, physiological control and environmental factors including temperature, photoperiod and water supply (Jackson, 1999; Sonnewald, 2001; Suttle, 2004a, 2004b; 2007; Sonnewald and Sonnewald, 2014; Li et al., 2018). It is generally accepted that tuber sprouting is accompanied by some metabolic changes including the degradation of starch and protein, the increases in soluble sugars and amino acids (Sonnewald, 2001; Hajirezaei et al., 2003; Viola et al.,

2007; Sonnewald and Sonnewald, 2014; Ferreira et al., 2017) and the variations of endogenous hormones (Davies and Viola, 1988; Debast et al., 2011; Hartmann et al., 2011), as well as some alterations in relevant gene transcripts and enzymatic reactions (Halford et al., 2005; Campbell et al., 2008; Liu et al., 2012, 2015a, 2015b).

Previous studies have shown that the supply of soluble carbohydrates is an important factor controlling the rate of potato tuber sprouting (Sonnewald and Sonnewald, 2014). At the onset of sprouting, initial energy demand for sprout outgrowth derives from pre-existing soluble hexoses and sucrose in the tuber parenchyma, however, soluble sugars are exhausted during the late stages of sprouting. Then starch degradation is triggered and accompanied by an accumulation of soluble sugars, that is, carbon supply of bud growth depends on starch breakdown in the later period (Davies and Ross, 1984, 1987; Biemelt et al., 2000; Hajirezaei et al., 2003; Viola et al., 2007; Sonnewald and Sonnewald, 2014). Late ethylene supplementation (at eye movement stage) delayed tuber sprouting, accompanied by a reduction of reducing sugars content and an increase in ABA levels (Foukaraki et al., 2014, 2016a; 2016b). There was cross-talk between ABA and other phytohormones, as well as with sugar metabolic pathways, which facilitated the onset of dormancy break and further sprouting (Alamar et al., 2017). Si et al. (2016) found that tuber sprouting time of the antisense

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transgenic lines transformed with the antisense *PPase* gene were delayed for 2 and 3 weeks, accompanied by a reduction of glucose, fructose and sucrose content. Li et al. (2017) analysed transcription and proteomics data from dormancy, sprouting, camphor inhibition and recovery sprouting samples, the results showed that camphor inhibited the processes of starch and sucrose metabolism, resulting in bud necrosis and delayed sprouting. By phloem-specific expression of yeast-derived cytosolic invertase (EC 3.2.1.26) in transgenic potato plants, phloem-transport of sucrose towards the developing bud was impaired in transgenic tubers during storage, accompanied by strong inhibition of tuber sprouting, simultaneously, starch degradation was accelerated and fructose levels increased slightly (Hajirezaei et al., 2003). A recent study revealed that three isoamylase (EC 3.2.1.68) isoforms (ISA1, ISA2 and ISA3) were involved in both starch granule formation and starch degradation in potato tubers. Simultaneous silencing of all three isoforms led to an early sprouting phenotype, accompanied by a reduction of starch content and a greater accumulation of sucrose in parenchyma cells below the outgrowing bud. At the same time, starch granule formation was impaired due to the silencing of three isoforms, accompanied by an accumulation of numerous small granules whose surface may be easier accessible for starch degrading enzymes to facilitate starch degradation (Ferreira et al., 2017). Thus, it can be seen that starch degradation plays a significant role in the regulation of tuber sprouting behavior. Nevertheless, very little is known about the regulation mechanism and specific starch degrading enzymes responsible for controlling potato tuber dormancy and sprouting.

Normally, starch degradation is either hydrolytic and phosphorytic, it is believed that the major pathway of starch breakdown occurs hydrolytically via α -amylase (EC 3.2.1.1) and β -amylase (EC 3.2.1.2) (Zeeman et al., 2010). Some functions for dormancy regulation of amylases have been reported in potato. Bailey et al. (1978) found that the activity levels of amylase and starch phosphorylase in the storage tubers increased dramatically after emergence from dormancy. Davies and Ross (1987) reported an increase in α -amylase activity accompanied by starch breakdown in sprouting potato tubers. Biemelt et al. (2000) found that the activities and transcript abundance of α -amylase and β -amylase increased in the sub-eye tissue of tubers after the onset of sprouting. Rentzsch et al. (2012) showed that gibberellins-induced dormancy release induced the expression of α -amylase, as well as α -amylase and β -amylase activity in tuber sprout and 'sub-eye' tissues, and low carvone concentrations enhanced the accumulation of amylase activity and α -AMY2-type α -amylase transcripts in sprouts. Hartmann et al. (2011) found by transcriptome analysis that the expression of micro.10377.c1 annotated as an α -amylase was strongly up-regulated at the time of sprouting during the sprout release assay.

Similar results were also reported from other species. Fincher (1989) showed that α -amylase is synthesized largely in the aleurone and scutellum, and secreted into the endosperm of germinating rice seeds. The expression sites of two α -amylase genes (*RAmy1A* and *RAmy3D*) were localized by in situ hybridization over five days of germination in rice (Ranjhan et al., 1992). In addition, two α -amylase genes (*HvAMY1* and *HvAMY2*) expressed during germination in barley (Bak-Jensen et al., 2010). A study also showed that suppressing rice α -amylase I-1 (*RAmy1A*) resulted in delayed seed germination, seedling growth and increased starch accumulation in the young leaf tissues under a sugar-supplemented condition (Asatsuma et al., 2005), implying the importance of α -amylase in sprouting control.

Our previous study had identified two α -amylases (StAmy1 and StAmy23) and seven β -amylases (StBAM1, StBAM3, StBAM4, StBAM5, StBAM7, StBAM8, and StBAM9) in the potato genome, of which StAmy23 was found to have the highest expression in tubers before sprouting, and the expression decreased after sprouting (Zhang et al., 2014). Additionally, Gausing and Kreiberg, (1994) found that these two α -amylase genes of potato were isolated from mRNA of sprouting tubers, but only Amy23 was detected in sprouts of potato tubers stored at 8 °C for 19 weeks. These results suggest that StAmy23 may play a role in

tuber sprouting control, of which the regulation mechanism is interesting to study.

In this study, we test the relationship of sprouting time with carbohydrates in *StAmy23*-RNAi transgenic potato tubers. Our results show that StAmy23 plays an important role in controlling potato tuber dormancy.

2. Materials and methods

2.1. Plant material and growth condition

Plants with RNA interference of *StAmy23* in *S. tuberosum* L. cv. Solara have been previously obtained (Ferreira et al., 2017). Solara and transgenic lines were maintained in tissue culture on MS medium supplemented with 4% (w/v) sucrose under a 12 h light and 12 h dark period at 20 °C. After propagation, the plants were grown at 18–25 °C in 24-cm-diameter plastic pots in the greenhouse (12 h light/12 h dark, light intensity ranged from 400 to 1000 $\mu\text{mol m}^{-2} \text{s}^{-1}$) at the National Centre for Vegetable Improvement (Central China), Huazhong Agricultural University (Wuhan, China).

2.2. Dormancy period test and sampling

The mature tubers were harvested and stored in darkness at room temperature, 12 tubers for each transgenic line were selected for dormancy period test. The experiment has been repeated for three seasons. The tubers for the dormancy period test were placed into boxes (5 L) and maintained in the dark at room temperature (23 ± 2 °C). A tuber is considered sprouted when it has a sprout of at least 1 mm long. The sprouting time was recorded at three-day interval until all tubers had sprouted. Samples from the unsprouted tuber cortex (the tissue within 5 mm below the bud eye) and pith (the tissue between 5 and 10 mm below the bud eye) were taken by punching a cork borer #2 in the tubers stored at room temperature for 6 weeks. The samples were cut into 1 mm slices, frozen in liquid nitrogen and stored at -70 °C for biochemical analysis.

2.3. RNA isolation and qRT-PCR

The frozen samples were ground in liquid nitrogen for RNA isolation as described previously (Liu et al., 2013). qRT-PCR was assayed as described by Liu et al. (2011). Potato gene *ef1a* (GenBank accession: AB061263) was used as a reference (Nicot et al., 2005). The qRT-PCR primers for *StAmy23* were used as before (Zhang et al., 2014). qRT-PCR was analyzed by CFX Connect™ Real-Time System (Bio-Rad, USA). The relative expression level was calculated by a comparative Ct method as described by Bio-Rad, and the significance was tested by Student's *t*-test.

2.4. Determination of sugar content, starch yield, starch content, phytoglycogen structure and starch granule size distribution

The measurements of glucose, fructose, sucrose and starch contents were performed as described previously (Müller-Röber et al., 1992). Reducing sugar content was calculated by the sum of glucose and fructose contents. Starch yield was computed according to Li et al. (2008) with modifications. Tuber starch content (TSC, percent fresh weight) was determined as described previously (Müller-Röber et al., 1992). Tuber yield (TY, gram) is measured by tuber weight. Tuber starch yield (TY, gram) is calculated by TSC*TY. For soluble starch and phytoglycogen structure measurement, sample processing was performed as described previously (Ferreira et al., 2017), soluble starch content was determined by measuring the amount of glucose released by treatment with α -amylase and amyloglucosidase (Smith and Zeeman, 2006). Phytoglycogen structure was determined as described previously (Streb et al., 2008). The data were presented as means of

three biological replicates, and significance was tested by Student's *t*-test on Microsoft Excel program (Microsoft Office, 2010). The starch granule size of potato tubers was determined according to Li et al. (2011) and Zhang et al. (2019). Three images were used to measure starch granule sizes. The starch granules were labelled manually, and the sizes were automatically measured in micrometers using the segmentation software ImageJ. The tiny granules are nearly invisible under the 20 × microscope objective and negligible in terms of starch biomass. Thus, starch granules with lengths equal to or greater than 5 μm were considered measurable for statistical calculations. The statistical analysis was done by Student's *t*-test.

3. Results

3.1. Silencing of *StAmy23* does not affect leaf starch content and starch yield in harvested tuber

To explore the roles of *StAmy23* in potato tuber sprouting control, three plants with RNA interference of *StAmy23* in potato cultivar Solara previously obtained by Ferreira (Ferreira et al., 2017) were employed for the functional test. All plants were grown at 12-h light/12-h dark, the transgenic lines showed normal plant morphology relative to the control (Hou et al., 2017). The leaf starch content showed a circadian change, starch accumulation reached peaks at the end of the light period (12 h) and was lowest at dawn (0 h). However, compared with the control, the variation of starch contents showed no significant difference in RNAi-*StAmy23* leaves (Fig. S1A). Meanwhile, the contents of glucose, fructose and sucrose were also determined in all the transgenic leaves and these exhibited no significant differences compared with the control (Figs. S1B–D). The starch yield per plant showed no significant differences in transgenic tubers except the line RNAi-*StAmy23*-15 (Fig. S2).

Interestingly, the starch granule size was slightly altered in some cases (Fig. 1). For example, higher amounts of small granules were accumulated in RNAi-*StAmy23*-15 and RNAi-*StAmy23*-18 lines relative to the control, but there were no obvious differences in RNAi-*StAmy23*-12 line (Fig. 1A). Furthermore, the mean length of the starch granule of RNAi-*StAmy23*-15 and RNAi-*StAmy23*-18 lines was smaller than the control, and there were no differences between RNAi-*StAmy23*-12 line and the control (Fig. 1B).

3.2. Silencing *StAmy23* leads to sprouting delay

The sprouting time of the tubers during storage after harvest was recorded. Strikingly, *StAmy23* silenced tubers exhibited a prolonged dormancy period (Fig. 2), the first visible sprouts appeared only after 67 days in the strongest line RNAi-*StAmy23*-12, and 100% sprouting was reached after 103 days. The weakest line RNAi-*StAmy23*-18 showed visible sprouting after 61 days, with 100% sprouting after 100 days. However, the first visible sprouts of the control (Solara) could be detected after 55 days and 100% sprouting was reached after 85 days. The results suggest that *StAmy23* silenced tubers started sprouting almost 1–2 weeks later than those of the control.

3.3. Reducing sugar content reduces in *StAmy23*-deficient potato tubers

The length of the dormancy period is regulated by an interplay between endogenous and environmental factors, and some changes happen in plant primary metabolism, especially in tissues below sprouting buds (Sonnewald and Sonnewald, 2014). In order to clarify the reason for dormancy lengthening, the expression level of *StAmy23* and sugar content of tuber cortex and pith tissue below the bud eye were investigated before the onset of sprouting (tuber stored at room temperature for 6 weeks). Compared with the control, the expression of *StAmy23* reduced about 54–77% in RNAi-*StAmy23* tuber cortex, and *StAmy23* by 64–80% in tuber pith (Fig. 3). Most of the *StAmy23*

silencing lines (Fig. 4A, B, C) accumulated less reducing sugar in tuber cortex and pith stored at room temperature for 6 weeks (in total and in glucose and fructose fractions) than in the control, but no obvious differences were detected in sucrose content (Fig. 4D).

3.4. Silencing of *StAmy23* impairs soluble starch degradation of the tuber cortex and pith tissue below the bud eye

The starch content of cortex and pith tissue below the bud eye in RNAi-*StAmy23* tubers stored at room temperature for 6 weeks was also investigated. Compared with the control, the soluble starch content exhibited an increase in RNAi-*StAmy23* tubers (Fig. 5B), and single component (phytglycogen and malto-oligosaccharides) showed an increase (Fig. 5C and D). The results suggest that silencing of *StAmy23* impair soluble starch degradation of tuber cortex and pith tissue below the bud eye. These results indicate that the sprouting delay might be due to a lower sugar content resulted from a reduction in soluble starch breakdown during storage.

3.5. Phytglycogen structure is slightly altered in *StAmy23*-silenced tuber cortex below the bud eye

To investigate whether the changes in soluble starch content were accompanied by the variation of glucan structures, the chain length distribution of soluble phytglycogen was determined in cortex tissue below the bud eye in RNAi-*StAmy23* tubers stored at room temperature for 6 weeks. The results showed that the chain length distribution of the phytglycogen from all RNAi-*StAmy23* lines was similar to each other in most cases (Fig. 6A), while *StAmy23*-silenced lines had a small decrease in the relative abundance of short glucan chains with a degree of polymerization (dp) between 4 and 5, and an increase in long chains (dp 30–38) compared with the control (Fig. 6B), which indicates that the phytglycogen is subject to gradual degradation by *StAmy23*. Hence, the accumulated soluble starch content can be interpreted that there were alterations in the phytglycogen structure.

4. Discussion

Sprouting is one major factor contributing to the nutritional and processing qualities loss resulting in remobilization of storage starch in potato (Sonnewald, 2001). Starch degradation can support energy for tuber dormancy breaking, and it is important to maintain sprout growth (Viola et al., 2007; Sonnewald and Sonnewald, 2014). However, genes related to starch degradation in tuber sprouting control remain to be clarified. In this study, we elucidated the role of α -amylase gene *StAmy23* involved to the starch breakdown in potato tuber dormancy and sprouting.

The potato genome encodes two α -amylases and seven β -amylases (Hou et al., 2017), but only the expression of α -amylase *StAmy23* was induced in tubers before sprouting and significantly lower after sprouting (Zhang et al., 2014). Our previous study showed that silencing of two major amylases (*StBAM1* and *SBAM9*) contributed to tuber starch degradation displayed normal sprouting behaviour in potato (Hou et al., 2017), while the present work demonstrated that silencing of *StAmy23* delayed tuber sprouting time compared with the control (Fig. 2), reflecting the function of *StAmy23* in tuber sprouting control. These suggest that *StAmy23* may be the main amylase in the regulation potato tuber sprouting.

It is generally accepted that α -amylase plays the main role in cereal grain starch degradation and germination (Zeeman et al., 2010). There is an abundant synthesis of α -amylase in the aleurone and scutellum, followed by secretion into the endosperm during germination (Fincher, 1989). Two α -amylase genes (*HvAMY1* and *HvAMY2*) were also expressed during germination in barley (Bak-Jensen et al., 2010). Moreover, seed germination and seedling growth were markedly delayed in transgenic rice with suppressed expression of α -amylase I-1 (Asatsuma

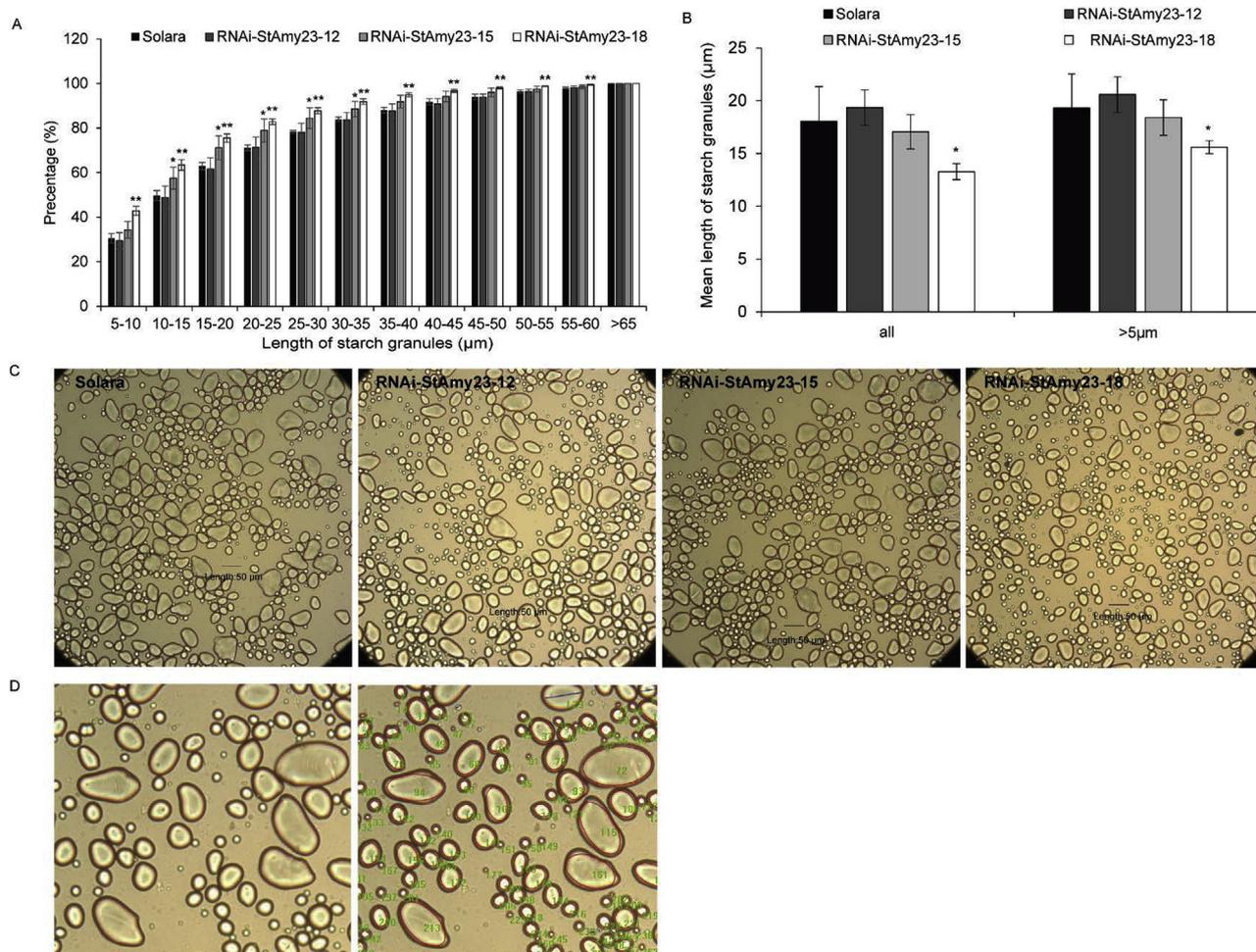


Fig. 1. Distribution and micrographs of starch granule size.

A, Relative percentage of the number of starch granules within a specific length range. B, Mean length of starch granules. Starch granules derived from harvested tubers. C, Micrographs of iodine-stained starch granules derived from RNAi-*StAmy23* and the control (Solara) tubers. D, Representative image displaying the image segmentation. Starch granules from 3 individual and harvested tubers were extracted, stained with iodine and analysed by light microscopy, starch granule size were measured by imaging and segmentation software ImageJ. The columns represent the mean values of three biological replicates and the bars indicate the standard deviation. * $P < 0.05$; ** $P < 0.01$ by Student's *t*-test.

et al., 2005). Similarly, our results showed that *StAmy23* did exert an influence on tuber dormancy, with sprouting delayed about 1–2 weeks in RNAi silencing lines (Fig. 2), which agrees well with previous reports.

Meanwhile, we found that delayed sprouting was accompanied by higher soluble malto-oligosaccharides and reduced accumulation of RS, glucose and fructose of cortex and pith tissue below the bud eye in *StAmy23* repression tuber stored at room temperature for 6 weeks (Figs. 4 and 5). These data are consistent with other reports in which induction of starch degradation and accumulation of soluble sugars were found in parenchyma cells during the late stages of sprouting (Viola et al., 2007), indicating the increase of starch turnover for maintaining sprout growth. Moreover, similar studies showed that the antisense *PPase* gene in transgenic potato plant delayed tuber sprouting time for two and three weeks compared with that of the wild-type, the delayed sprouting was accompanied by reduced glucose, fructose and sucrose content (Si et al., 2016). Vreugdenhil (2007) reported that modifying carbohydrate metabolism affected tuber dormancy and sprouting. In addition, it has been shown that tuber sprouting is associated with sugar and hormone metabolic pathway, which facilitated the onset of dormancy break and further sprouting (Hartmann et al., 2011; Sonnewald and Sonnewald, 2014; Foukaraki et al., 2014, 2016a; 2016b; Alamar et al., 2017). Li et al. (2017) reported by transcriptome

and proteomics analyses that camphor delayed tuber sprouting by inhibiting the process of starch and sucrose metabolism. These suggest that carbohydrate metabolism may be an important factor controlling the rate of potato tuber sprouting. Hence, we speculate that sprouting delay may be due to soluble sugars shortage resulted from the reduced breakdown of soluble starch in *StAmy23*-silenced tubers. In agreement with the observation that delayed seed germination and seedling growth was overcome by supplementation of sugars in silenced α -amylase I-1 lines in rice (Asatsuma et al., 2005). There were several studies (Sonnewald, 2001; Hajirezaei et al., 2003; Viola et al., 2007; Ferreira et al., 2017) suggested that sucrose plays a crucial role in tuber sprouting control, sucrose content increased especially in tissues below sprouting buds. Surprisingly, compared with the control, no concordant differences were detected in sucrose content in cortex and pith tissue below the bud eye in *StAmy23*-silenced tuber stored at room temperature for 6 weeks (Fig. 4D), one possible explanation for this may be due to different sampling time, and this will need to be further investigated.

In addition, the soluble phytyglycogen from *StAmy23* repression tuber cortex had also altered chain length distributions (Fig. 6), showing less proportion of very short chains (dp 4 to 5) and elevated long chains (dp 30 to 38). Previous studies have also shown that *StAmy23* mainly acted on soluble phytyglycogen (Hou et al., 2017). Collectively, these data led to assume that the reduced dormancy period

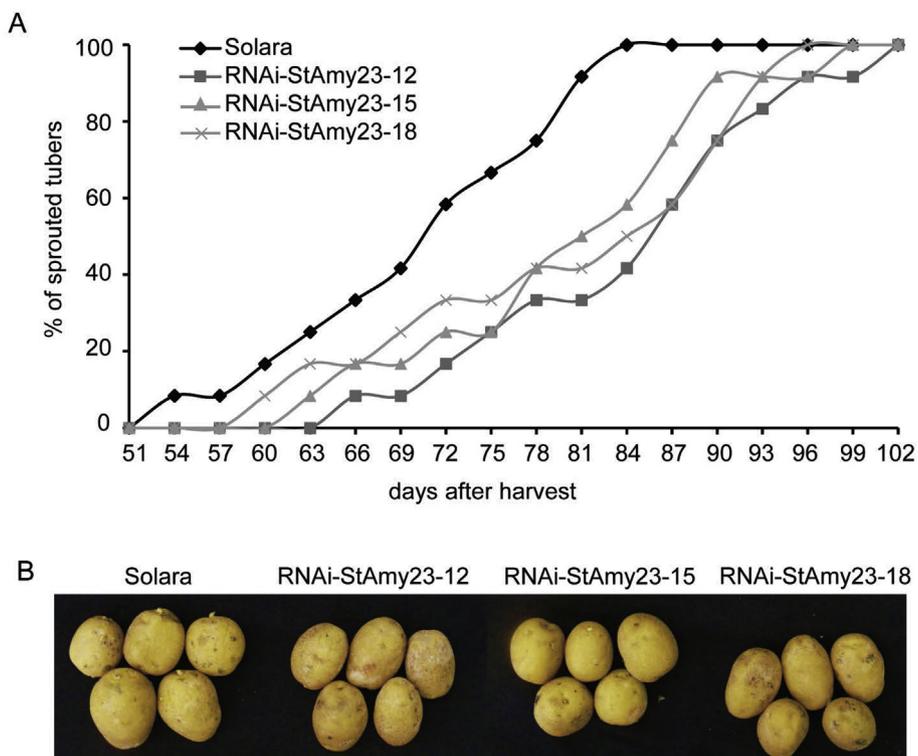


Fig. 2. Impact of *StAmy23* silencing on tuber sprouting. A, The percentage of tuber sprouting during storage after harvest. B, Photographs of RNAi-*StAmy23* tubers and control (Solara) at 80 days after harvest.

of *StAmy23*-silenced potato tubers is mediated by the sugar accumulation, *StAmy23* might operate to stimulate sprouting by hydrolyzing soluble phytoglycogen to ensure supply of sugars and play a predominant role during tuber dormancy.

5. Conclusion

Our study elucidated the role of *StAmy23* in controlling potato tuber dormancy and sprouting. *StAmy23* RNAi transgenic potato tubers displayed a delayed sprouting phenotype. Carbohydrate contents and phytoglycogen structure analysis showed that *StAmy23* may stimulate

sprouting by hydrolyzing soluble phytoglycogen during tuber dormancy.

Author contributions

B. Song and U. Sonnewald designed the research. J. Hou, B. Song, T. Liu, H. Zhang and X. Peng performed plant growth, leaves and tuber treatment. J. Hou, B. Song and S. Reid performed dormancy period, gene expression, sugar content, starch content, phytoglycogen structure and granule size distribution analysis. J. Hou, B. Song and T. Liu analyzed the data. J. Hou, B. Song, U. Sonnewald, S. Reid, J. Du and K. Sun

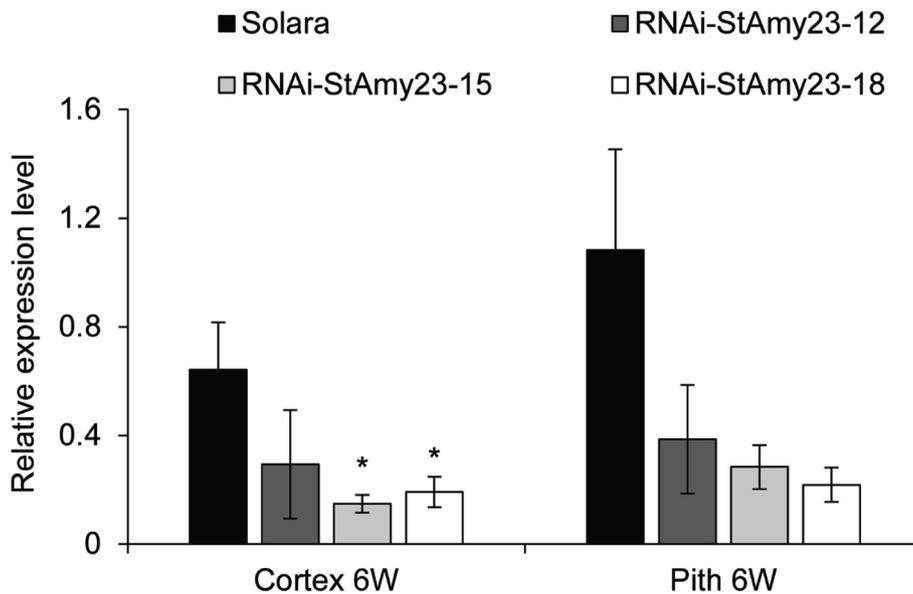


Fig. 3. The transcripts of *StAmy23* in cortex and pith tissue below the bud eye from RNAi-*StAmy23* tubers stored at room temperature for 6 weeks.

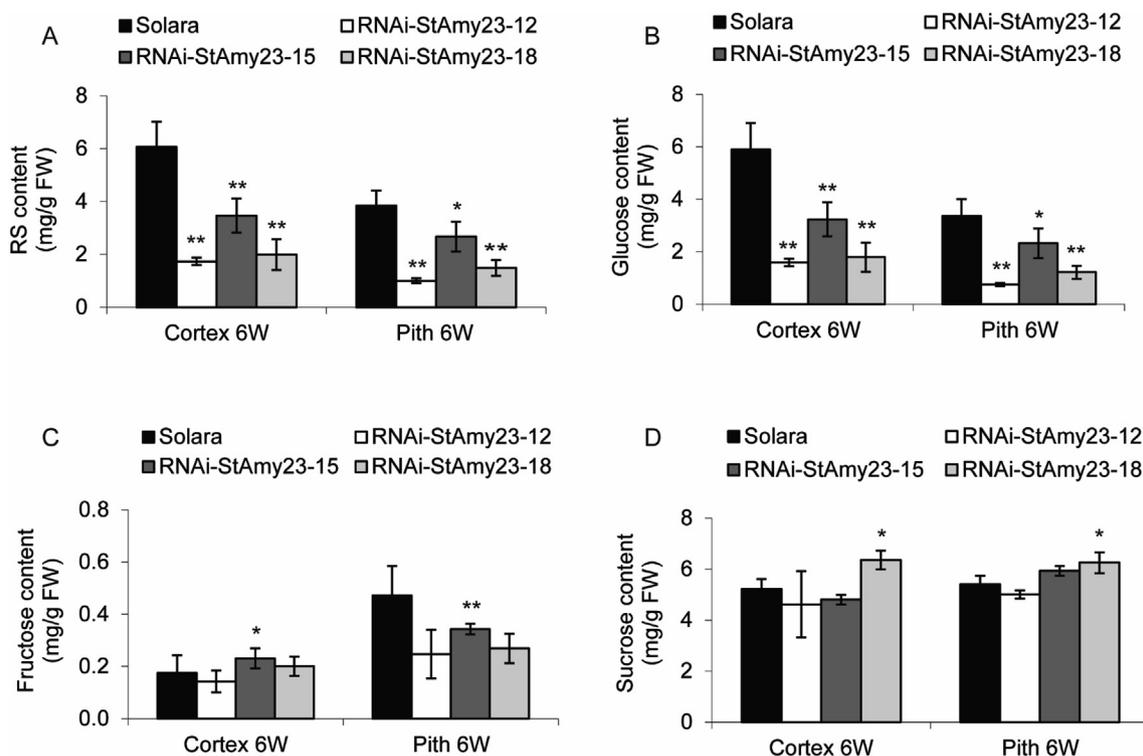


Fig. 4. The sugar content of cortex and cortex and pith tissue below the bud eye from RNAi-StAmy23 tubers stored at room temperature for 6 weeks. A, RS content. B, Glucose content. C, Fructose content. D, Sucrose content. The columns represent the mean values of three biological replicates and the bars indicate the standard deviation. *P < 0.05; **P < 0.01 by Student's t-test.

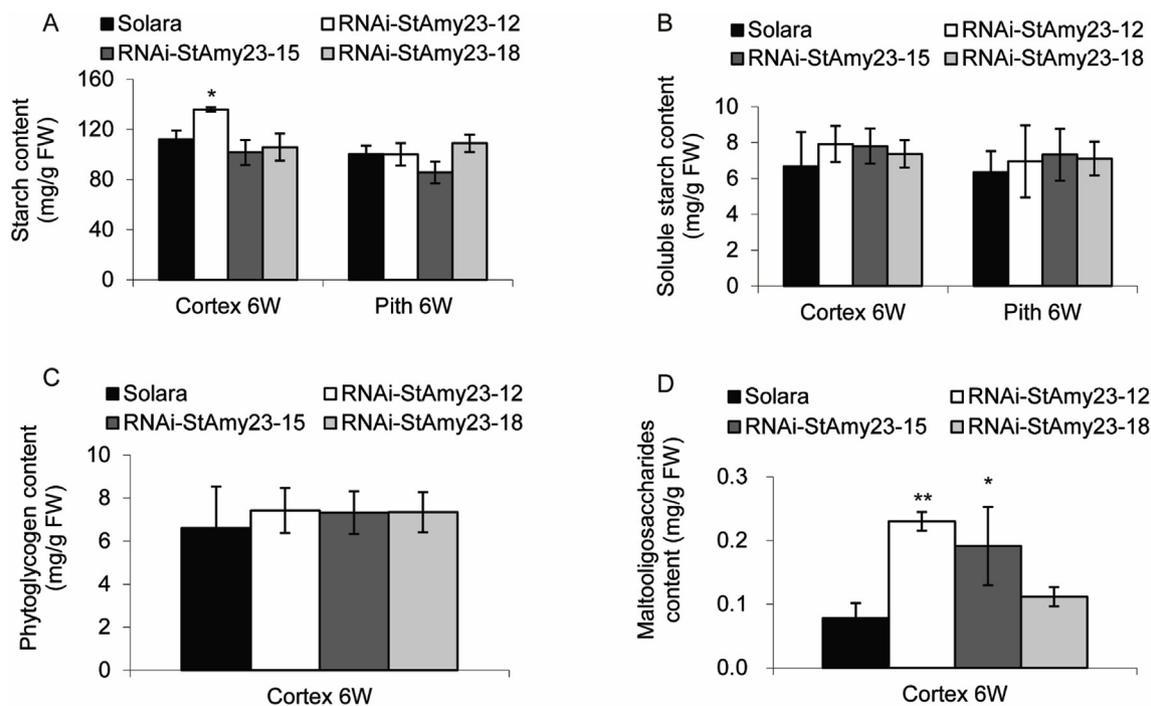


Fig. 5. The starch content of cortex and cortex and pith tissue below the bud eye from RNAi-StAmy23 tubers stored at room temperature for 6 weeks. A, Starch content. B, Soluble starch content. C, Phytyglycogen content. D, Malto-oligosaccharides content. The columns represent the mean values of three biological replicates and the bars indicate the standard deviation. *P < 0.05; **P < 0.01 by Student's t-test.

wrote the article.

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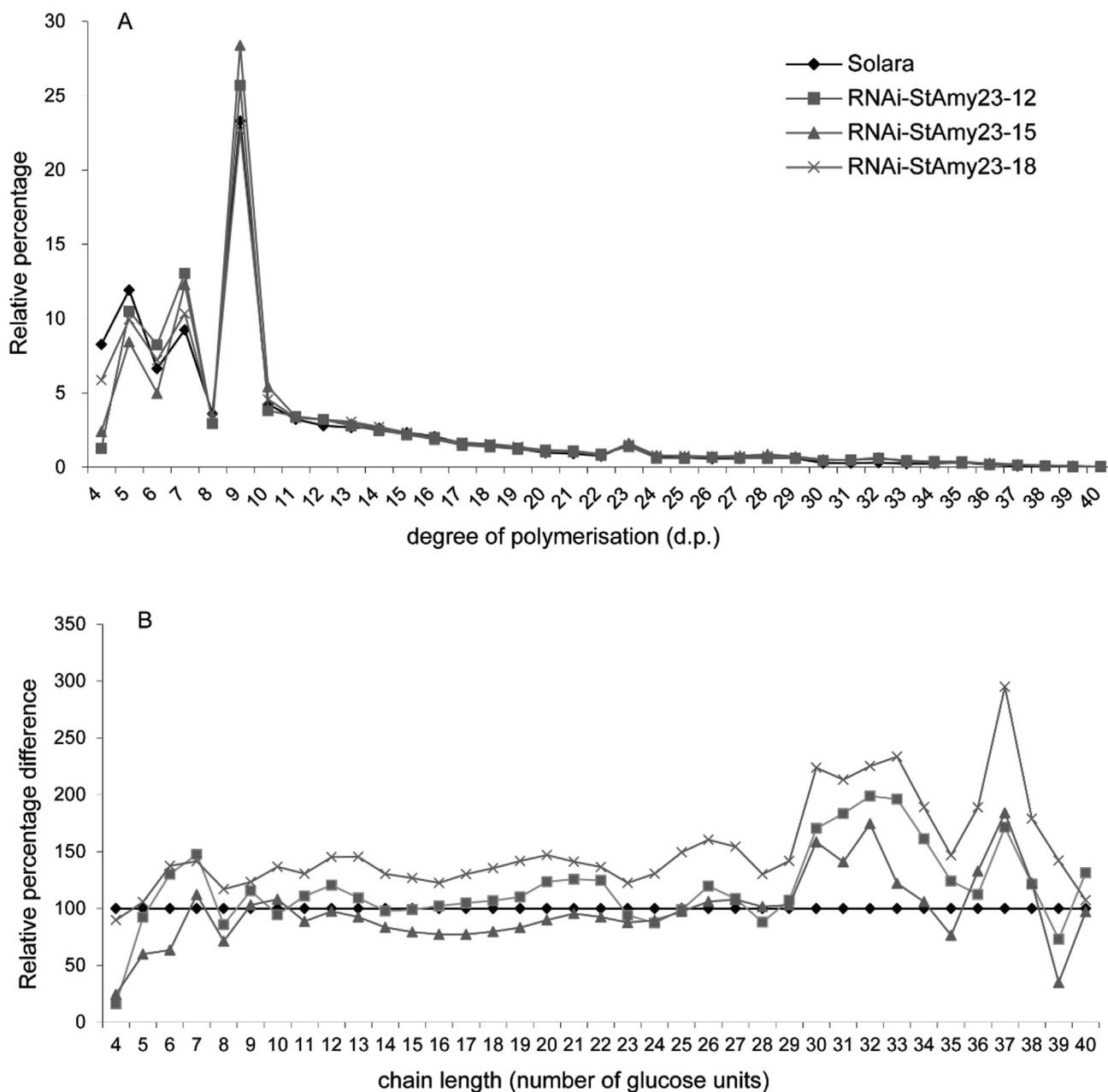


Fig. 6. Relative chain length distributions of phytyglycogen from RNAi-StAmy23 tuber cortex tissue below the bud eye. A, The percentage of chain length distribution. B, Difference plots derived by subtracting the relative percentage values of the control from those of RNAi-StAmy23 lines (The control is taken as 100%). The cortex was sampled from RNAi-StAmy23 and the control tubers stored at room temperature for 6 weeks.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.plaphy.2019.03.044>.

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