Constitutive expression of *SlTrxF* increases starch content in transgenic *Arabidopsis*

F.B. WANG^{1*}, W.L. KONG², Y.R. FU³, X.C. SUN¹, X.H. CHEN¹, and Q. ZHOU¹

School of Life Science and Food Engineering, Huaiyin Institute of Technology, Huai'an 223003, Jiangsu, P.R. China¹

Departments of Microbiology and Immunology, University of Rochester Medical Center, Rochester, New York 14642, USA²

School of Landscape Architecture, Zhejiang A & F University, Lin'an 311300, Zhejiang, China³

Abstract

The plastidic thioredoxin F-type (TrxF) protein plays an important role in plant saccharide metabolism. In this study, a gene encoding the TrxF protein, named *SlTrxF*, was isolated from tomato. The coding region of *SlTrxF* was cloned into a binary vector under the control of 35S promoter and then transformed into *Arabidopsis thaliana*. The transgenic *Arabidopsis* plants exhibited increased starch accumulation compared to the wild-type (WT). Real-time quantitative PCR analysis showed that constitutive expression of *SlTrxF* up-regulated the expression of ADP-glucose pyrophosphorylase (AGPase) small subunit (*AtAGPase-S1* and *AtAGPase-S2*), AGPase large subunit (*AtAGPase-L1* and *AtAGPase-L2*) and soluble starch synthase (*AtSSS I*, *AtSSS II*, *AtSSS III* and *AtSSS IV*) genes involved in starch biosynthesis in the transgenic *Arabidopsis* plants. Meanwhile, enzymatic analyses showed that the major enzymes (AGPase and SSS) involved in the starch biosynthesis exhibited higher activities in the transgenic plants compared to WT. These results suggest that *SlTrxF* may improve starch content of *Arabidopsis* by regulating the expression of the related genes and increasing the activities of the major enzymes involved in starch biosynthesis.

Additional key words: ADP-glucose pyrophosphorylase, saccharide biosynthesis, *Solanum lycopersicum*, soluble starch synthase, tomato.

Introduction

Starch, as a main source of nutrition in human and animal diet, is a major storage saccharide in plants and plays a fundamental role in plant survival and adaptation to varying environmental conditions (Blennow *et al.* 2013, Skryhan *et al.* 2015). In plants, starch is an insoluble glucan composed of two glucose polymers: amylose and amylopectin. Amylose mainly comprises linear chains that are linked by α -1,4 O-glycosidic bonds, whereas amylopectin is highly branched and contains 5 - 6 % of α -1,6 O-glycosidic bonds to generate glucan branches of various lengths (Delvallé *et al.* 2005). Four major enzymes are involved in starch biosynthesis: ADP-glucose pyrophosphorylase (AGPase), starch synthase (SS), starch branching enzyme (SBE), and starch

debranching enzyme (DBE) (Fujita *et al.* 2006, Szydlowski *et al.* 2009).

Starch metabolism is regulated at many levels, including allosteric regulation by redox potential, protein-protein interactions, and protein phosphorylation (Kötting *et al.* 2010). Redox regulation, which has been widely studied in the context of starch synthesis, is also associated with the coordination of enzymes involved in starch degradation (Kötting *et al.* 2010). The redox regulation of the activity of AGPase (Geigenberger 2011) and β -amylase 1 (BAM1) (Sparla *et al.* 2006, Valerio *et al.* 2010) is well known.

Thioredoxins (Trxs) are ubiquitous small proteins (*ca.* 12 kDa) belonging to the oxidoreductase family of

Submitted 25 January 2016, *last revision* 8 July 2016, *accepted* 13 July 2016.

Abbreviations: AGPase - ADP-glucose pyrophosphorylase; Hyg - hygromycin; ORF - open reading frame; qPCR - quantitative PCR; SSS - soluble starch synthase; Trx - thioredoxin; VC - control vector; WT - wild-type.

Acknowledgments: This research was financially supported by the Natural Science Foundation of Jiangsu Province of China (BK2013256), the National Spark Plan Project of China (2014GA69002), and the Support Project of Jiangsu Provincial Department of Agriculture (BE2012445).

* Corresponding authors; fax: (+86) 517 83591100, e-mail: wangfeibing1986@163.com

enzymes and play a fundamental role in regulating multiple cellular processes (Gelhaye *et al.* 2005, Meyer *et al.* 2005). The active site of Trxs is located within the characteristic thioredoxin fold and contains two reactive cysteine (Cys) residues in a conserved CXXC motif. The plastidic Trxs F-type proteins have been shown to act *in vitro* as reductants for many of the redox-regulated enzymes involved in starch metabolism, suggesting that the redox regulation that occurs in response to light signals is similar to that controlling photosynthetic enzymes (Schürmann and Buchanan 2008). The *TrxF* gene has been shown to be involved in starch metabolism. Overexpression of *NtTrxF* increases starch

accumulation in the leaves of tobacco (Sanz-Barrio *et al.* 2013). Consistently, knockout of *AtTrxF1* expression decreases starch content in the leaves of *Arabidopsis* mutants (Thormählen *et al.* 2013).

Although the function of *Trxs* from other plant species has been well studied, the regulatory role of *SITrxF1* (accession No. XM_004239601) from tomato in increasing starch accumulation still remains unknown. In this work, the coding region of *SITrxF* from tomato was cloned into a binary vector under the control of 35S promoter and constitutively expressed in *Arabidopsis*. The starch biosynthesis in transgenic plants was studied.

Materials and methods

Cloning and sequence analysis of the tomato *SITrxF* gene: Tomato (*Solanum lycopersicum* Mill.) cv. Zhongshu No. 4 was employed for *SITrxF* gene cloning in this study. The plants were grown in a greenhouse under a 14-h photoperiod and a temperature of 25 °C.

Total RNA was extracted from the leaves of 30-d-old plants with the *RNAprep* pure kit (Tiangen Biotech, Beijing, China). RNA samples were reverse-transcribed according to the instructions of *Quantscript* reverse transcriptase kit (Tiangen Biotech). Based on the sequence of *SITrxF* (accession No. XM_004239601), we designed one gene-specific primer of reverse transcription PCR (RT-PCR) (Table 1 Suppl.) to obtain full-length cDNA sequence. PCR was performed with an initial denaturation 94 °C for 3 min, followed by 35 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min and final extension at 72 °C for 10 min. The PCR products were separated on a 1.0 % (m/v) agarose gel. Target DNA bands were recovered by gel extraction, then cloned into PMD19-T (*TaKaRa*, Beijing, China), and finally transformed into competent cells of *Escherichia coli* strain DH5 α . White colonies were checked by PCR and the positive colonies were sequenced (*Invitrogen*, Beijing, China).

The full-length cDNA of *SITrxF* was analyzed by *BLAST* on the National Center for Biotechnology Information (*NCBI*) website (<http://www.ncbi.nlm.nih.gov/>). For multiple sequence alignment analysis, the amino acid sequence of *SITrxF* and *Arabidopsis* *AtTrxF* homologs retrieved from *NCBI* were aligned using the *DNAMAN* software (*Lynnon Biosoft*, Quebec, Canada). The theoretical molecular mass and isoelectric point (pI) were calculated using *ProtParam* tool (<http://web.expasy.org/protparam/>). The conserved domain of *SITrxF* was scanned by the *InterProScan* program (<http://www.ebi.ac.uk/Tools/pfa/iprscan/>).

Generation of transgenic *Arabidopsis* plants: *Arabidopsis thaliana* L. (ecotype Columbia-0) was used

as a wild type (WT) and a model plant to investigate the functions of *SITrxF*. The coding region of *SITrxF* was cut out from the PMD19-T vector with the restriction enzymes *Bam*H I and *Sac* I, and then inserted into the same enzyme sites in pCambia1301 to create the plant expression vector pCambia1301-*SITrxF* under the control of the cauliflower mosaic virus (CaMV) 35S promoter and the nopaline synthase (NOS) terminator (Fig. 1). This vector also contains β -glucuronidase (*gusA*) and hygromycin resistance (*hpt* II) genes driven by the CaMV 35S promoter (Fig. 1). Both pCambia1301-*SITrxF* and the control vector (VC) pCambia1301 were transformed into the *Agrobacterium tumefaciens* L. strain LBA4404 cells by the electroporation method for *Arabidopsis* transformation (Lou *et al.* 2007). Transgenic plants were produced according to methods described previously (Zhang *et al.* 2006). Transformants were selected based on their resistance to hygromycin (Hyg). Putative transformant seeds were germinated on agar-solidified Murashige and Skoog (1962; MS) medium containing 25 mg dm⁻³ Hyg. Positive transgenic seedlings were grown in pots containing a mixture of soil, *Vermiculite* and humus (1:1:1, v/v/v) for T₂ and T₃ seed selection. The incubation and growth conditions of *Arabidopsis* were the same as described previously (Zhang *et al.* 2006).

PCR analysis of transgenic plants: The presence of *SITrxF* in hygromycin-resistant plants was assessed by PCR analysis using specific primers (Table 1 Suppl.) to amplify fragments of the *hpt* II coding sequence. DNA was first extracted from *Arabidopsis* leaves according to the instructions of *EasyPure* plant genomic DNA kit (*Transgen*, Beijing, China). PCR amplifications were performed with an initial denaturation at 94 °C for 3 min, followed by 35 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min and final extension at 72 °C for 10 min. The PCR products were separated by electrophoresis on a 1.0 % (m/v) agarose gel.

Starch content and AGPase and SSS activities: Seedlings were grown on MS medium for 2 weeks and transferred to pots containing a mixture of soil, *Vermiculite* and humus (1:1:1, v/v/v). Plants were grown in a growth chamber at a temperature of 22 °C, a 14-h photoperiod, an irradiance of 120 $\mu\text{mol m}^{-2} \text{s}^{-1}$, and an air humidity of 75 %. Leaves of 4-week-old plants were harvested at 10:00 - 11:00. Starch was extracted with 80 % ethanol and quantified as described by Smith and Zeeman (2006). All treatments were performed in triplicate.

The activities of starch biosynthetic enzymes (AGPase and SSS) in the leaves of four-week-old transgenic and WT plants was performed according to the method described by Nakamura *et al.* (1989). One unit of activity (AGPase and SSS) was defined as the formation of 1 nmol ADP per min at 30 °C.

Expression analysis of the related genes: The expression of *SlTrxF* and starch biosynthesis related genes was analyzed by real-time quantitative (q) PCR. Transgenic and WT plants were grown in pots for 4 weeks under above mentioned conditions. Total RNA

was extracted from the leaves of these plants and reverse-transcribed as mentioned above. The cDNA solution was used as templates for PCR amplification with gene specific primers (Table 1 Suppl.). *Arabidopsis actin* gene (accession No. NM112764) was used as an internal control (Li *et al.* 2013) (Table 1 Suppl.).

PCR amplifications were conducted by *ABI PRISM 7500* (7500 Fast real-time PCR system, V2.0.1, CA, USA, and respective software) using *SYBR Green PCR Master Mix* (Tiangen Biotech). The amplifications were performed with an initial denaturation at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Quantification of gene expression was done with comparative C_T method (Schmittgen and Livak 2008). All experiments were repeated three times and each data represents the average of three experiments.

Statistical analyses: All experiments were repeated three times and the data presented as the mean $\pm$ standard error (SE). Where applicable, data were analyzed by Student's *t*-test in a two-tailed analysis. Values at $P < 0.05$ or < 0.01 was considered to be statistically significant differences.

Fig. 1. Schematic diagram of the binary construct pCambia1301-*SlTrxF*. LB - left border, RB - right border, *hptII* - hygromycin phosphotransferase II gene, *SlTrxF* - tomato thioredoxin F-type gene, *gusA* - β -glucuronidase gene, 35S - cauliflower mosaic virus (CaMV) 35S promoter, 35S T - CaMV 35S terminator, NOS T - nopaline synthase terminator.

Fig. 2. Gene structure and protein alignment analyses. A - Structure of the *SlTrxF* gene and *SlTrxF* protein. The *SlTrxF* protein contains a thioredoxin F-type domain. B - Amino acid sequence alignment of *SlTrxF* with *Arabidopsis* *AtTrxF1* and *AtTrxF2*. The *SlTrxF* protein contains 4 α -helices and 4 β -strands.

Results

SITrxF (accession No. XM_004239601) was cloned by reverse transcription (RT)-PCR. *SITrxF* contains a 549 bp open reading frame (ORF), encoding a polypeptide of 182 amino acids, with a molecular mass of 19.89 kDa and a theoretical isoelectric point (pI) of 9.26. Sequence analysis via the *InterProScan* program showed that the SITrxF protein contained a thioredoxin F-type domain

(Fig. 2A). A *BLASTP* search indicated that the amino acid sequence of SITrxF shared 56.22 and 58.92 % amino acid identity to *Arabidopsis thaliana* AtTrxF2 (AT5G16400) and AtTrxF1 (AT3G02730), respectively (Fig. 2B). Tertiary structure prediction analyses revealed that ZmTrxF contained 4 α -helices and 4 β -strands (Fig. 2B).

Fig. 3. Molecular confirmation of transgenic plants. *A* - PCR analyses of *SITrxF* expressing *Arabidopsis* plants. M - DL2000 DNA marker, W - water as negative control, P - plasmid pCambia1301-*SITrxF* as positive control, WT - wild type, VC - control vector, #1 - #5 - different transgenic lines. *B* - Expressions of *SITrxF* in different transgenic lines. The *Arabidopsis actin* gene was used as an internal control. Means $\pm$ SEs ($n = 3$), ** indicate significant differences from WT at $P < 0.01$, determined by Student's *t*-test.

Fig. 4. Starch content in the leaves of 4-week-old WT, VC, and transgenic plants. Means $\pm$ SEs ($n = 3$). * and ** indicate significant differences from WT at $P < 0.05$ and < 0.01 , respectively.

The ORF of *SITrxF* was ectopically expressed in *Arabidopsis* (Col-0, WT) using the binary vector pCambia1301-*SITrxF* (Fig. 1). Five independent transgenic lines constitutively expressing *SITrxF* (T_1 generation) were obtained by Hyg resistance selection, named #1 - #5, respectively, and their progenies (T_3 generation) were generated. PCR analyses of genomic

DNA confirmed the successful integration of transgene (Fig. 3A). Real time qPCR analyses showed that the highest expressions of *SITrxF* were observed in transgenic lines #2, #3, and #5, while no transgene expression was observed in the control vector (VC) and WT (Fig. 3B). Therefore, transgenic lines #2, #3, and #5 were selected for further analyses.

Two-week-old WT, VC, and lines #2, #3, and #5 were grown in pots for 4 weeks and no significant differences in growth were observed between WT, VC, and the transgenic plants (data not shown). However, the starch content in the leaves of these plants was different. The starch content in *SITrxF* expressing plants increased 54 - 98 % compared to that in WT (Fig. 4), whereas no significant difference was observed between VC and WT plants (Fig. 4).

To dissect how expression of *SITrxF* increased starch content in transgenic plants, the transcriptions of 8 starch biosynthetic genes in WT, VC, and transgenic plants were examined by real time qPCR (Fig. 5). Expressions of genes related to starch biosynthesis pathway, such as AGPase small subunits (*AtAGPase-S1* and *AtAGPase-S2*), AGPase large subunits (*AtAGPase-L1* and *AtAGPase-L2*)

and soluble starch synthases (*AtSSS I*, *AtSSS II*, *AtSSS III*, and *AtSSS IV*) were all up-regulated in transgenic plants (Fig. 5). These results indicate that *SlTrxF* might be involved in the regulation of starch biosynthesis.

The activities of major enzymes (AGPase and SSS) involved in starch biosynthesis were also investigated in the leaves of WT, VC, and transgenic plants (Fig. 6). The

activities of these enzymes were significantly higher in transgenic plants compared to WT (Fig. 6). The increases in enzyme activities in transgenic plants were consistent with the increased transcription of their corresponding genes. All these results demonstrate that *SlTrxF* significantly enhanced the activity AGPase and SSS in transgenic plants.

Fig. 5. The expression of starch biosynthesis genes in the leaves of 4-week-old WT, VC, and *SlTrxF* expressing transgenic plants. The *Arabidopsis actin* gene was used as an internal control. The results are shown as relative values with respect to WT, which was set to 1.0. Means $\pm$ SEs ($n = 3$). * and ** indicate significant differences from WT at $P < 0.05$ and < 0.01 , respectively.

Discussion

Starch is the most abundant storage polysaccharide in higher plants. The *TrxF* gene has been shown to be involved in starch accumulation (Sanz-Barrio *et al.* 2013, Thormählen *et al.* 2013). Here, we successfully cloned *SlTrxF*, a homolog of *AtTrxF1* from tomato, which has similar protein structure to *AtTrxF1* and *AtTrxF2* (Fig. 2). Constitutive expression of *SlTrxF* in *Arabidopsis*

significantly increased starch content in transgenic plants (Fig. 4).

The *TrxF* gene is related to saccharide metabolism, functions in post-translational redox activation of AGPase and starch biosynthesis (Ballicora *et al.* 2000, Geigenberger *et al.* 2005, 2011, Sanz-Barrio *et al.* 2013, Thormählen *et al.* 2013). The study with the knockout

mutant of *AtTrxF1* showed that loss of function of *AtTrxF1* resulted in decreased redox activation of AGPase and starch accumulation in the mutant (Thormählen *et al.* 2013). Sanz-Barrio *et al.* (2013) reported that overexpression of *NtTrxF* increased the AGPase activity, leading to increased starch content in transgenic tobacco. Consistently, AGPase activity was also significantly increased in the *SITrxF* expressing *Arabidopsis* plants (Fig. 6). In addition, the expression of *AtAGPase-S1* and *AtAGPase-S2*, encoding AGPase small subunit, and *AtAGPase-L1* and *AtAGPase-L2*, encoding AGPase large subunits, were up-regulated in transgenic plants (Fig. 5). Thus, constitutive expression of *SITrxF* up-regulated the expression of *AtAGPase* and increased the activity of AGPase, which further modulated starch metabolism (Fig. 7).

The higher starch content is related to the increased expression of starch biosynthesis genes (Delvallé *et al.* (Delvallé *et al.* 2005, Fujita *et al.* 2006, Szydlowski *et al.*

Fig. 6. The activities of ADP-glucose pyrophosphorylase (AGPase) and soluble starch synthase (SSS) in the leaves of 4-week-old WT, VC, and *SITrxF* expressing plants. Means $\pm$ SEs ($n = 3$), ** indicate significant differences from WT at $P < 0.01$.

2009). A large body of evidence has illustrated that up-regulation of these genes could increase starch accumulation in plants (Burton *et al.* 2002, Bustos *et al.* 2004, Roldan *et al.* 2007, Szydlowski *et al.* 2009, Jiang *et al.* 2013). In our study, a significant increase of SSS activity was also observed in transgenic plants (Fig. 6) and consistently, genes *AtSSS I*, *AtSSS II*, *AtSSS III*, and *AtSSS IV* were systematically up-regulated in transgenic plants (Fig. 5). Therefore, expression of *SITrxF* up-regulated the expression of *AtAGPase* and increased the activity of AGPase, which further increased the expression of the genes and the activity of the major enzymes involved in starch biosynthesis, leading to increased starch accumulation in transgenic plants (Fig. 7).

Fig. 7. A proposed model of the regulatory network of *SITrxF* in starch accumulation. Biosynthesis pathway is shown with solid arrows and regulatory interactions with broken arrows. $\uparrow$ indicates up-regulation of the enzyme encoding genes.

Taken together, the *SITrxF* gene was successfully isolated from tomato. Constitutive expression of *SITrxF* significantly increased starch accumulation in transgenic *Arabidopsis* plants. Therefore, *SITrxF* could play an important role in starch metabolism, suggesting its great potential in developing high starch-accumulating plants.

References

- Ballicora, M.A., Frueauf, J.B., Fu, Y., Schürmann, P., Preiss, J.: Activation of the potato tuber ADP-glucose pyrophosphorylase by thioredoxin. - *J. biol. Chem.* **275**: 1315-1320, 2000.
- Blennow, A., Jensen, S.L., Shaik, S.S., Skryhan, K., Carciofi, M., Holm, P.B., Hebelstrup, K.H., Tanackovic, V.: Future cereal starch bioengineering cereal ancestors encounter gene technology and designer enzymes. - *Cereal. Chem.* **90**: 274-287, 2013.
- Burton, R.A., Jenner, H., Carrangis, L., Fahy, B., Fincher, G.B., Hylton, C., Laurie, D.A., Parker, M., Waite, D., Wegen, S.V., Verhoeven, T., Denyer, K.: Starch granule initiation and growth are altered in barley mutants that lack isoamylase activity. - *Plant J.* **31**: 97-112, 2002.
- Bustos, R., Fahy, B., Hylton, C.M., Seale, R., Nebane, N.M., Edwards, A., Martin, C., Smith, A.M.: Starch granule initiation is controlled by a heteromultimeric isoamylase in potato tubers. - *PNAS* **101**: 2215-2220, 2004.
- Delvallé, D., Dumez, S., Wattedled, F., Roldán, I., Planchot, V., Berbezy, P., Colonna, P., Vyas, D., Chatterjee, M., Ball, S., Mérida, A., D'Hulst, C.: Soluble starch synthase I: a major determinant for the synthesis of amylopectin in *Arabidopsis thaliana* leaves. - *Plant J.* **43**: 398-412, 2005.
- Fujita, N., Yoshida, M., Asakura, N., Ohdan, T., Miyao, A., Hirochika, H., Nakamura, Y.: Function and characterization of starch synthase I using mutants in rice. - *Plant Physiol.* **140**: 1070-1084, 2006.
- Geigenberger, P., Kolbe, A., Tiessen, A.: Redox regulation of carbon storage and partitioning in response to light and sugars. - *J. exp. Bot.* **56**: 1469-1479, 2005.
- Geigenberger, P.: Regulation of starch biosynthesis in response to a fluctuating environment. - *Plant Physiol.* **155**: 1566-1577, 2011.
- Gelhay, E., Rouhier, N., Navrot, N., Jacquot, J.P.: The plant thioredoxin system. - *Cell. mol. Life Sci.* **62**: 24-35, 2005.
- Jiang, T., Zhai, H., Wang, F.B., Yang, N.K., Wang, B., He, S.Z., Liu, Q.C.: Cloning and characterization of a carbohydrate metabolism-associated gene *IbSnRK1* from

- sweetpotato. - *Sci Hort.* **158**: 22-32, 2013.
- Kötting, O., Kossmann, J., Zeeman, S.C., Lloyd, J.R.: Regulation of starch metabolism: the age of enlightenment? - *Curr. Opin. Plant Biol.* **13**: 321-329, 2010.
- Li, X., Ma, H., Huang, H., Li, D., Yao, S.: Natural anthocyanins from phytoresources and their chemical researches. - *Natur. Prod. Res.* **27**: 456-469, 2013.
- Lou, X.M., Yao, Q.H., Zhang, Z., Peng, R.H., Xiong, A.S., Wang, K.K.: Expression of human hepatitis B virus large surface antigen gene in transgenic tomato. - *Clin. Vaccine Immunol.* **14**: 464-469, 2007.
- Meyer, Y., Reichheld, J.P., Vignols, F.: Thioredoxins in *Arabidopsis* and other plants. - *Photosynth. Res.* **86**: 419-433, 2005.
- Murashige, T., Skoog, F.: A revised medium for rapid growth and bioassays with tobacco tissue cultures. - *Physiol. Plant* **15**: 473-497, 1962.
- Nakamura, Y., Yuki, K., Park, S.Y., Ohya, T.: Carbohydrate metabolism in the developing endosperm of rice grains. - *Plant Cell Physiol.* **30**: 833-839, 1989.
- Roldan, I., Wattebled, F., Lucas, M.M., Delvalle, D., Planchot, V., Jimenez, S., Perez, R., Ball, S., D'Hulst, C., Merida, A.: 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 J.* **49**: 492-504, 2007.
- Sanz-Barrio, R., Corral-Martinez, P., Ancin, M., Segui-Simarro, J.M., Farran, I.: Overexpression of plastidial thioredoxin *f* leads to enhanced starch accumulation in tobacco leaves. - *Plant Biotechnol. J.* **11**: 618-627, 2013.
- Schmittgen, T.D., Livak, K.J.: Analyzing real-time PCR data by the comparative C_T method. - *Nat. Protoc.* **3**: 1101-1108, 2008.
- Schürmann, P., Buchanan, B.B.: The ferredoxin/thioredoxin system of oxygenic photosynthesis. - *Antioxid. Redox Signal.* **10**: 1235-1274, 2008.
- Skryhan, K., Cuesta-Seijo, J.A., Nielsen, M.M., Marri, L., Mellor, S.B., Glaring, M.A., Jensen, P.E., Palcic, M.M., Blennow, A.: The role of cysteine residues in redox regulation and protein stability of *Arabidopsis thaliana* starch synthase 1. - *PLoS ONE* **10**: e0136997, 2015.
- Smith, A.M., Zeeman, S.C.: Quantification of starch in plant tissues. - *Nat. Protoc.* **1**: 1342-1345, 2006.
- Sparla, F., Costa, A., Lo Schiavo, F., Pupillo, P., Trost, P.: Redox regulation of a novel plastid-targeted beta-amylase of *Arabidopsis*. - *Plant Physiol.* **141**: 840-850, 2006.
- Szydlowski, N., Ragel, P., Raynaud, S., Lucas, M.M., Roldan, I., Montero, M., Munoz, F.J., Ovecka, M., Bahaji, A., Planchot, V., Pozueta-Romero, J., D'Hulst, C., Merida, A.: Starch granule initiation in *Arabidopsis* requires the presence of either class IV or class III starch synthase. - *Plant Cell* **21**: 2443-2457, 2009.
- Thormählen, I., Ruber, J., Von Roepenack-Lahaye, E., Ehrlich, S.M., Massot, V., Hummer, C., Tezycka, J., Issakidis-Bourguet, E., Geigenberger, P.: Inactivation of thioredoxin *f1* leads to decreased light activation of ADP-glucose pyrophosphorylase and altered diurnal starch turnover in leaves of *Arabidopsis* plants. - *Plant Cell Environ.* **36**: 16-29, 2013.
- Valerio, C., Costa, A., Marri, L., Issakidis-Bourguet, E., Pupillo, P., Trost, P., Sparla, F.: Thioredoxin-regulated beta-amylase (BAM1) triggers diurnal starch degradation in guard cells, and in mesophyll cells under osmotic stress. - *J. exp. Bot.* **62**: 545-555, 2010.
- Xiong, A.S., Yao, Q.H., Peng, R.H., Duan, H., Li, X., Fan, H.Q., Cheng, Z.M., Li, Y.: PCR-based accurate synthesis of long DNA sequences. - *Nat. Protoc.* **1**: 791-797, 2006.
- Xiong, A.S., Yao, Q.H., Peng, R.H., Li, X., Fan, H.Q., Cheng, Z.M., Li, Y.: A simple, rapid, high-fidelity and cost-effective PCRbased two-step DNA synthesis method for long gene sequence. - *Nucl. Acids Res.* **32**: e98, 2004.
- Zhang, X., Henriques, R., Lin, S.S.: *Agrobacterium*-mediated transformation of *Arabidopsis thaliana* using the floral dip method. - *Nat. Protoc.* **1**: 641-646, 2006.