StASR 2 Overexpression Decreases Salicylic Acid Accumulation Leading to Lower Stomatal Conductance in *Nicotiana tabacum*

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Abstract:

Plant ASRs (ABSCISIC ACID STRESS RIPENING) contains conserved domains in response to ABA and water deficiency, which function on fruit development and drought response. Overexpression of ASR leads to stoma close, enhancing plant tolerance to drought. However, it is unknown yet that how ASR regulates stoma close or open. In this study, morphological and physiological phenotypes were assayed with transgenic lines overexpressing StASR 2 cDNA from potato in Nicotiana tabacum. Data analysis showed that overexpressing StASR 2 resulted in decrease on stoma conductance and transpiration ratio, attributing to a low salicylic acid accumulation level. Although accumulations of chlorophyll and ATP intensified in StASR 2 overexpression lines, net photosynthesis ratio was attenuated owing to reduced stoma conductance. Meanwhile, StASR 2 overexpression leaflets no long appeared sensitivity to ABA treatment and no significant changes on leaf ABA contents was detected between StASR 2 overexpression and control. The result indicates that less salicylic acid accumulation mediates involvement of StASR 2 overexpression in stomatal close rather than ABA.

Keywords: ASR, gene, stoma, salicylic acid, ATP

Introduction

There are 5 genes encoding ASRs (*ABSCISIC ACID STRESS RIPENING*) transcripts in *Solanum lycopersicum* genenome. Early study found that all of these genes appear high level expression during tomato fruit development. It was showed in the late study that ASRs differentially express in all plant organs (Golan et al.2014; Dominguez et al. 2015).

ASR amino acid sequences from different sources shares a commons ABA/WDS domain in response of both ABA and water-deficit stress (WDS). The existence of this domain indicated that ASR has the potential function of responding to water shortage. At the same time, ASR was positively regulated by sucrose and glucose and involved in the accumulation of ABA during fruit maturation and seed germination (Jia et al.2016: Li et al.2017), which also supported ASR function in response to water deficiency.

Overexpressing of tomato ASR1 enhanced survival rates of tomato seedling under water stress, whereas suppression of ASR1 expression displayed sensitive to water deficiency(Golan et al.2014; Dominguez et al. 2015) . Drought induced high expression of *OsASR*1 and *OsASR*3, which were the main rice ASR members induced by the stress(Joo et al.2014). The loss-of-function of *OsASR*5 resulted in both lowering of leaf relative water

contents and sensitive to drought stress (Li et al.2017). Therefore, ASRs are a water stress response genes and positively regulated drought tolerance.

More importantly, overexpression of *OsASR*5 led to stomata closure and low stomatal conductance. The result is identical with tomato ASR1, which positively regulates of drought tolerance(Golan et al.2014; Li et al.2017). The opening and closing of stomata was regulated by multiple stresses (Merilo et al.2018). The relationship between ASR expression and stomatal conductance provides an explanation, of which ASR involves in response to water deficiency by regulation of stomatal conductance. But how ASR associates with stomatal conductance is unknown yet.

Materials and Methods

Materials

Transgenic seedlings and in vitro plantlets of *Nicotiana tabacum* grow under the conditions of 16-h day/8-h night, temperature 20-25 ºC, light intensity 100μmol · m^{-2} s^{-1}, relative humidity 60 ~ 80%. After reaching 3-5 cm in highth , the plantlets were transplanted to the Pins medium produced by Pindstrup Group in Denmark. Then keep medium water content of 40-50%. After growing for 30d, young leaves were collected and stored at -80℃, and used for further assays. The transformation vector pC-35S, containing 35S promoter was preserved in our laboratory.

Methods

Transformation of StASR 2 into Nicotiana tabacum.

In terms of DNA sequence DMG400006663 in the potato genome database annotated as abscisic stress-ripening protein 2 (*StASR* 2), 336 bp full-length cDNA of *StASR* 2 was synthesized and cloned in pUC57 vector by Sangon Biotech (Shanghai) Co. Ltd. After sequencing confirmed, *StASR* 2 cDNA was subcloned into pC-35S with *Sal* I and *Sac* I digestion to generate pC-*StASR*-2. Following protocol as described by Liu (Liu et al.2012), *StASR*-2 was transformed into *Nicotiana tabacum*. PCR and GUS positive lines were selected.

RT-qPCR

With primer pair ASR-2F:5' CCACCACAAGAACAAGGAGGAAGA 3' and ASR-2R:5' CGGCAGCAACAGCACCAAGT 3' and the real-time fluorescence quantitative PCR system (Light Cycler® 480II, Roche,Germany), RT-qPCR was performed under the reaction conditions as follows: after initial denaturation of 95 ℃ 5min, 95 ℃ 15 s to 62 ℃ 30 s, 45 cycles, melting curve is 65 ℃ -95 ℃, reference gene is L25.

Assay on photosynthesis parameters , chlorophyll and ATP contents Stomatal conductance, transpiration rate and net photosynthetic rate were determined by GFS3000 portable photosynthesis system produced from WALZ Company of Germany. The chlorophyll content was measured by the SPAD-502Plus type chlorophyll meter produced by KONICA MINOLTO Company in Japan. The unit of chlorophyll content is mg·mL^{-1}.

Using Plant ATP contents assay kit produced by Shanghai Enzyme-Linked Biotechnology Co., Ltd. to complete the determination of ATP content. The unit of ATP content is nmol·L^{-1}. According to the instructions, the content of salicylic acid and the content of PRDX5 in leaves were determined by using the salicylic acid content determination kit which produced by Shanghai Qiyi Biotechnology Co., Ltd. The unit of salicylic acid content is ng·mL^{-1} and PRDX5 is pg·mL^{-1}.

Assay on ABA stress response

Set 0,1,2 and 3 μmol·L^{-2} ABA concentration gradient, prepare 0.8% MS solid medium plate and select the third new leaf of each lines, wash with 10% NaCl and 70% ethanol , the explants of 0.5cm × 0.5cm were cut out under sterile condition and inoculated on flat plate. After 14 days of culture under the above conditions, the number of yellowing in explants were statisticed on.The 80% area yellowing count of explants as etiolated explants. According to formula: survival rate =1- yellow flower explant percentage. Three plates were set up for each concentration treatment and each plate was inoculated with 25 explants.

Data analysis

Statistical analysis of the assay data was completed using the Statistical Program for Social Sciences (SPSS V19.0) t- test .

Sequence analysis

With BLAST tools in Uniprot (http://www.uniprot.org) and Solanaceae genome(https://solgenomics.net/) databases, *StASR* 2 nucleic acid and amino acid sequences were retrieved. Sequence alignment was completed with Clustal Omega (http://www.clustal.org/). TargetP 1.1(http://www.cbs.dtu.dk/services/TargetP/) and InterPro database (http://www.ebi.ac.uk/interpro/) were performed to predict domain.

Results

StASR 2 contains ABA-WDS domain

Potato StASR 2 cDNA, 443 bp in length contains a 337-bp ORF and encoding predicted peptide composed of 111 amino acids. 4 homologous sequences with a similarity of 80 to 100% were identified by BLAST on potato genome database with *StASR 2* amino acid sequence. 4 genes mapped on chromosome 4 of potato genome, and each gDNA contained two exons. No one of the four genes was annotated as function known. Blast in the Uniprot database hits function-known ASR2 of tomato (*Solanum lycopersicum*), which is only one of ASRs annotated. Therefore, *StASR* 2, 100% similar with gene ID DMG400006663 endoing DMP400011776 was named.

Blast in *Nicotiana tabacum* genome database with predicted *StASR* 2 amino acid sequence, 4 function-known homologous sequences, with a similarity of 78% to 84% were identified. *StASR*3 amino acid sequence retrieved the Arabidopsis genome database and no homologous sequence was found. Alignment of potato and common tobacco ASRs, as showed in Fig.1 displayed a high conservity of ASRs from 2 species. The Uniprot database predicted that *StASR* 2 expresses in nucleus. InterPro (66.0) database search showed that *StASR* 2 sequence was mainly composed of ABA-WDS (PF02496). In addition, function-

known tomato ASR2 was annotated as "response to stress". Therefore, ABA-WDS domain containing *StASR* 2 may also function in response to stress.

Overexpression of StASR3 in positive lines

According to *StASR* 2 mRNA accumulating assayed by RT-qPCR with PCR and GUS staining positive lines, three *StASR* 2 overexpression lines were selected as shown in Figure 2, referring to them as *stasp*-1, *stasp*-3 and *stasp*-5. The relative expression fold of *StASR* 2 mRNA of each line was significantly higher than that of WT ($P < 0.01$), and relative expression fold average was 2.85 times higher than WT, indicating that the overexpression of target gene *StASR*3 was achieved.

Overexpression of StASR3 reduces stomatal conductance

As shown in Figure 3A, the stomatal conductances of the three *StASR*3 overexpression lines were significantly lower than WT ($p < 0.01$), with average value 33% lower than WT, suggesting that overexpression *StASR* 2 leads to decrease of stomatal conductance. The transpiration rate of three *StASR 2* overexpression lines was significantly lower than WT ($p < 0.01$) as showed in Figure 3B, and the mean value of transpiration rate was 49% lower than WT. The transpiration rate decreased along with overexpression of *StASR* 2.

As shown in Figure 3C, the net photosynthetic rate of three *StASR*3 overexpressed lines was significantly lower than WT ($p < 0.01$), and its mean value was 26% lower than control. Take all together, it can be concluded that CO_2 absorption slows down along with decreased stomatal conductance resulting from overexpression of *StASR* 2. The result was consistent with the decrease in stomatal conductance of *OsASR*5 overexpression in rice (Li et al.2017).

Overexpression of StASR3 reduces leaflets sensitivity to ABA

As shown in figure 3D, under different concentrations of ABA stress, there was no significant difference in the survival rate of explants between control and *StASR* 2 overexpression lines at 0 to 1 $\mu mol \cdot L^{-2}$ ABA concentration. With the increase of ABA concentration from 1 $\mu mol \cdot L^{-2}$ to 3 $\mu mol \cdot L^{-2}$, the survival rate of explants with overexpression of *StASR* kept increasing, but the survival rate of explants of control lines decreased sharply. The results showed that overexpression of *StASR* 2 enhanced the tolerance of transformed lines to ABA stress, in other words, overexpression of *StASR*3 decreased the sensitivity of transformed lines to ABA stress.

Overexpression of StASR3 increases chlorophyll and ATP accumulation

The chlorophyll contents of three *StASR*3 overexpression lines was significantly higher than WT ($p < 0.01$) , as showed in Figure 4A, and the average content was 16% higher than the control. Chlorophyll accumulation increased as a result of *StASR* 2 overexpression. As showed in Figure 4B, the average value of ATP contents is 57% higher than the control. The results indicated that overexpression of *StASR 2* led to increased accumulations of ATP and chlorophyll.

Photo-phosphorylation is one of the pathways of ATP synthesis. Theotically, rising chlorophyll accumulation gives rise to more active photo-phosphorylation due to enhanced light energy absorption. The result of increased ATP accumulation suggests that *StASR* 2 overexpression positively effects on chlorophyll accumulation. Up to date, *StASR*3

overexpression leads to increased chlorophyll and ATP accumulation has not been reported in the literature.

StASR 2 overexpression reduces salicylic acid accumulation

The data analysis on physiological phenotypes showed that the salicylic acid content of StASR3 overexpression lines was significantly lower than WT ($p < 0.01$), as shown in Figure 5A. The mean value of 0.87 ±0.14 mmol was only 44% of that of control, which was 1.96 ±0.15 mmol. The results showed that StASR3 overexpression reduced the accumulation of salicylic acid. The data analysis of peroxide-reducing protein PRDX5 showed that the PRDX5 content in StASR3 overexpression lines was significantly lower than WT ($p < 0.01$) and its mean value is 31% lower than the control as shown in Figure 5B. The results indicated that StASR 2 overexpression decreased redox level due to less PRDX5 accumulation. There was no significant difference in abscisic acid contents between StASR3 overexpression lines and control, indicating that StASR3 overexpression had no effect on abscisic acid accumulation.

Take all above together, the study can conclude as following. StASR3 overexpression reduced the accumulation of endogenous salicylic acid in leaves, leading to lower stomatal conductance and redox level and the sensitivity of leaves to ABA stress. In spite of the increase of chlorophyll and ATP accumulation induced by the overexpression of StASR 2, but the decrease of stomatal conductance still results in a low net photosynthesis rate. Therefore, it is concluded that the accumulation of endogenous salicylic acid was reduced by the overexpression of StASR 2, which subsequently lowers stomatal conductance and net photosynthesis rate as a consequence.

Discussion

It has been reported recently that the application of salicylic acid can increase chlorophyll accumulation in rice under drought stress (Tang et al. 2017; Kim et al.2018; Agnihotri et al.2018). This is identical with overexpression of *StASR*3 increased chlorophyll accumulation and light energy absorption, thus enhancing ATP accumulation as described in this study. The reasons for how salicylic acid need to be further studied. Previous studies indicated that accumulation of endogenous salicylic acid enhanced drought tolerance of Arabidopsis thaliana and winter wheat (Okuma et al.2014; Sedaghat et al. 2017). 0.5 mmol salicylic acid treatment on *Dianthus superbus* under salt stress increased stomatal conductance and chlorophyll content (Ma et al.2017). It seems that less salicylic acid accumulation, led to a higher stomatal conductance was reduced by salt stress, which reversed by salicylic acid treatment.

Recent study clearly showed that low salicylic acid concentration reduced the sensitivity of plant to stress and high salicylic acid concentration induced high level of oxidative stress, which resulted in decreased stress tolerance (Miura et al. 2014; Mimouni et al. 2016; El-Esawi et al. 2017). This result is identical with it drawn from our study. The findings of this study provide the reason for why low concentration of salicylic acid reduced sensitivity to stress.

No change of ABA accumulation was detected in the over-expression of *StASR* 2, suggesting that *StASR* 2 as well as low stomatal conductance induced by *StASR* 2 is

nothing to do with ABA.

In this paper, *StASR 2* regulated stomatal conductance and photosynthesis through the accumulation of salicylic acid. The results not only laid a foundation for further study of drought tolerance gene ASR involved in water stress response, but also enriched the knowledge on stomatal opening and closing regulation. The results of this study provide a new explanation for the results of previous studies.

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Fig.1 Alignment of *StASR*3 and homologs from *Nicotiana tabacum*

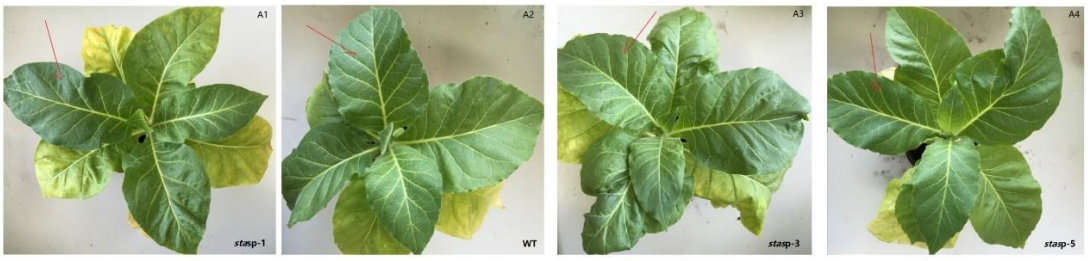

Fig. 2 phenotype comparison on *StASR*3 overexpression lines and WT

(Note: Red arrow showed position for sampling)

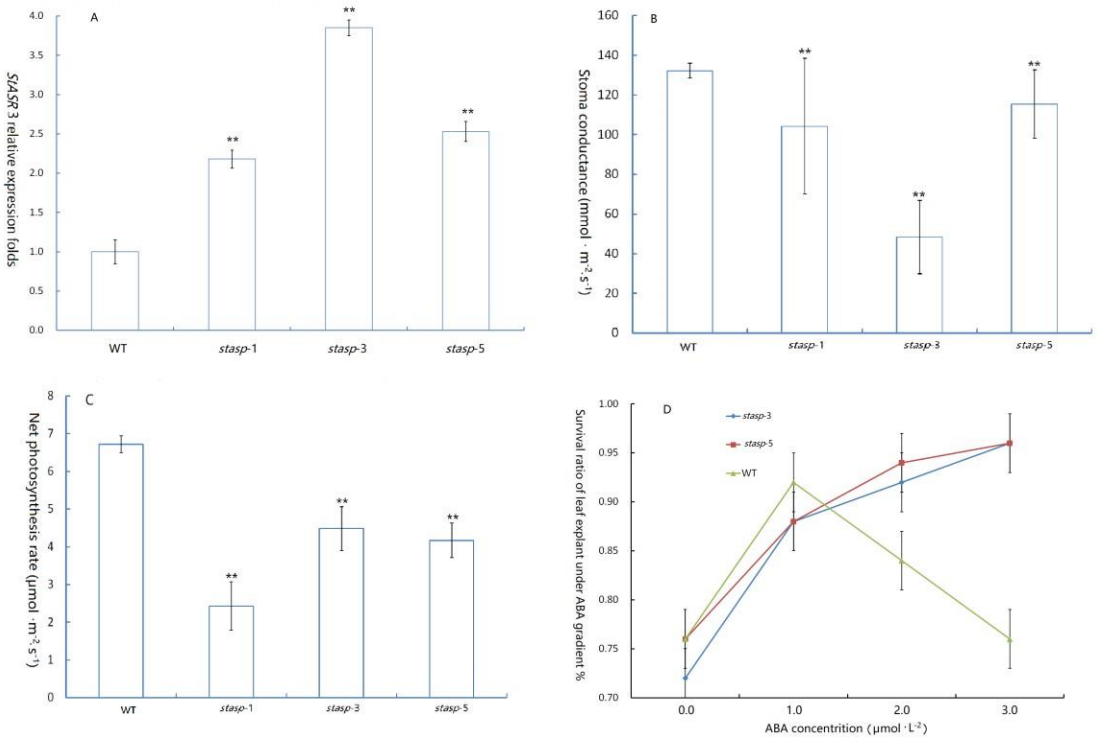

Figure 3 Assay results on *StASR* 2 mRNA accumulation and photosynthesis parameters of *StASR* 2 overexpression lines and WT

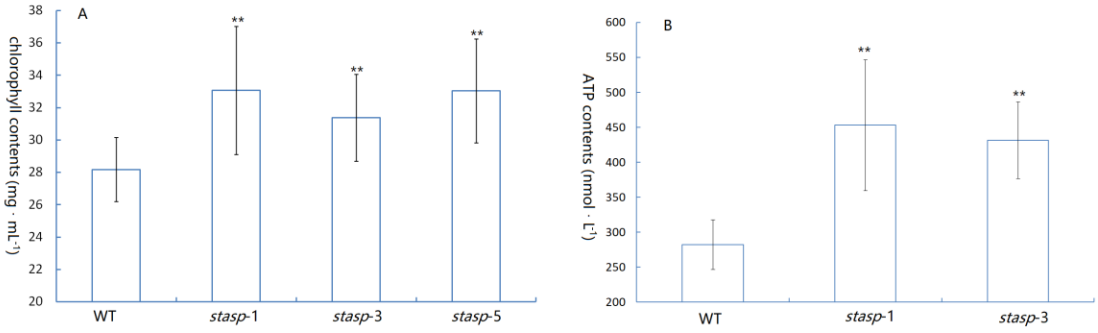

Fig. 4 Assay on chlorophyll and ATP contents in *StASR*3overexpression lines and WT

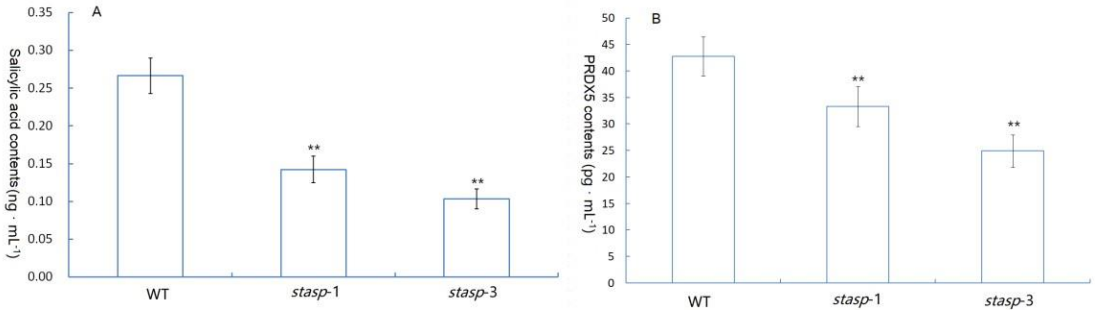

Fig. 5 Assay on salicylic acid and ATP contents in lines of *StASR3* overexpression and WT