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Overexpression of *StGA2ox1* Gene Increases the Tolerance to Abiotic Stress in Transgenic Potato (*Solanum tuberosum* L.) Plants

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Abstract

It has been known that *GA2ox* is one kind of key enzyme gene in the gibberellin synthesis pathway, which plays important regulatory roles throughout plant whole growth and development. In this article, one of the *GA2ox* family genes, designated *StGA2ox1*, was isolated from potato (*Solanum tuberosum* L.). The full length of cDNA is 1005 bp, and the cDNA corresponds to a protein of 334 amino acids; this protein was classified in a group with *NtGA2ox3* based on multiple sequence alignments and phylogenetic characterization. A plant expression vector pCAEZ1383-*StGA2ox1* was established. qRT-PCR showed that the expression of *RD28*, *DREB1*, *WRKY1*, and *SnRK2* genes in *StGA2ox1* transgenic plant is higher than that in non-transformed control under dehydration, low temperature conditions, and abscisic acid treatments. Overexpression of *StGA2ox1* cDNA in transgenic potato plants exhibited an improved salt, drought, exogenous hormone, and low temperature stress tolerance in comparison to the non-transformed plant. The enhanced stress tolerance may be associated with the subsequent accumulation of proline osmoprotectant in addition to a better control of chlorophyll, carotenoids, and water loss. These data suggest that the *StGA2ox1* is involved in the regulation of plant growth and tolerance in potato by regulating the synthesis of gibberellin.

Keywords Potato · *GA2ox1* · Cloning · Generation and transformation · Abiotic stress

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Introduction

Gibberellin (GA) is a large family of terpenoids; it plays an important role in regulating the growth and development of whole plant life. As an important hormone in plant, gibberellin involved in controlling a variety development of plants, including the germination of seed, elongation of stem, growth of root, stretching of leaf, development of epidermal hair, growth of pollen tube, development of flower and fruit, etc. [1, 2]. Gibberellin biosynthetic pathway can be divided into three stages, these key enzymes involved in the synthesis of gibberellin mainly include copalyl pyrophosphate synthase (*CPS*), ent-kaurene synthase (*KS*), ent-kaurene oxidase (*KO*), GA-20 oxidase (*GA20ox*), and GA-2 oxidase (*GA2ox*) [3, 4].

GA2ox, also called GA2 beta hydroxylase, is a multigene family in plant. *GA2ox* plays a very important regulating role in the GA biosynthetic pathway, it can catalyze the C_2 to hydroxylation in GA biosynthetic pathway, this process can promote the transformation of activated GA_1 and GA_4 to inactive gibberellin GA_8 and GA_{34} [5]. *GA2ox* catalyzes bioactive GAs or their immediate precursors to inactive forms; therefore, playing a direct role in determining the levels of bioactive GAs [6].

So far, *GA2ox* have been characterized in many plant species including *Phaseolus coccineus* [7], pea (Martin et al. 1999), poplar [8], *Arabidopsis thaliana* [9], rice [10], and spinach [11]. And a higher level of *GA2ox* expression has been reported to correlate with low concentrations of endogenous bioactive GAs [6, 10, 12, 13]. Schomburg identified two genes in Arabidopsis (*AtGA2ox7* and *AtGA2ox8*), using an activation-tagging mutant screen, GA levels in both activation-tagged lines were reduced significantly, and the lines displayed dwarf phenotypes typical of mutants with a GA deficiency [10]. The PcGA2ox1 gene was introduced into *Solanum melanocerasum* and *S. nigrum* (Solanaceae) by *Agrobacterium*-mediated transformation, and the transgenic plants exhibited a range of dwarf phenotypes associated with a severe reduction in the concentrations of the biologically active GA_1 and GA_4 [12]. In liliaceous monocotyledon *Tricyrtis* sp. of overexpressing the *GA2ox* gene, all transgenic plants exhibited dwarf phenotypes and had small or no flowers, and smaller, rounder, and darker green leaves; meanwhile, the endogenous levels of bioactive GAs, GA_1 and GA_4, largely decreased in transgenic plants as shown by liquid chromatography–mass spectrometry (LC–MS) analysis, and the level also correlated with the degree of dwarfism [6].

Environmental stresses often produce certain effect on the growth of plants. Under these stresses, plants often change the morphology and physiological function by adjusting the hormone metabolism and signal, to improve the stress tolerance. In fact, these changes are implemented by modulating the expression of response genes [14–17]. In recent years, research has shown that GA also involved in the physiological stress and abiotic stress. Under salt stress, the GA content of *Arabidopsis thaliana* decreased, while the accumulation of negative regulatory factor DELLA protein can improve the resistance of plants to salt stress [18, 19].

Potato (*Solanum tuberosum* L.) is one of the most important global food crop, following rice, wheat, and maize [20]. It is not only an important food source but also an important material in the starch-processing industry, a source of animal feed that makes use of potato vines and a potentially important resource in medicine owing to the compounds found in its seeds [21]. So, improving the yield and quality of potato is one of the important works for researchers. For GA plays an important regulating role in the whole growth and development of plant, and *GA2ox1* is a key enzyme gene in the synthesis of GA, so it is one of the ways to improve the development and tolerance of potato plant by researching the GA relative genes.

In this paper, we report the cloning and characterization of *StGA2ox1* gene and construction of the plant expression vector pCAEZ1383-*StGA2ox1*. The transgenic lines were obtained and their trail was observed. The expression of transgene in plants was investigated under abiotic stress conditions and ABA treatment. Overexpression of *StGA2ox1* in transgenic potato plants Mingshu 1 can improve the tolerance to dehydration stress in comparison to the non-transformed plant.

Results

Isolation and Alignment Analysis of the *StGA2ox1* cDNA

A fragment of 1200 bp was isolated from the potato material (Q9), and the full-length cDNA sequence, designated as *StGA2ox1*, is 1005 bp (Fig. 1). This *StGA2ox1* cDNA correspond to a protein of 334 amino acids, including 39 of strongly basic amino acids (+) (K, R), 36 of strongly acidic amino acid (−) (D, E), 115 of hydrophobic amino acids (A, I, L, F, W, V), and 87 of polar amino acid (N, C, Q, S, T, Y). The molecular weight of StGA2ox1 encoding amino acids is 37,687.48 Da, and the isoelectric point is 8.324.

Compared with prediction sequence of *GA2ox1* on NCBI (LOC102605134), *GA2ox1* gene sequences of Q9 are changed in 113, 276, 471, 860, 897, and 983 base position. Corresponding encoding amino acids also have some changes, such as the 38th amino acid that is from asparagine (N) into serine (S), the 287th is from glutamic acid (E) to glycine (G), the 328th is from valine (V) to alanine (A) (Fig. 2a). After the change, asparagine (N) and serine (S) both belong to the polar amino acid, valine (V) and alanine (A) both belong to the hydrophobic amino acids, and glutamic acid (E) belongs to the strongly acidic amino acids, glycine (G) belongs to polar amino acid.

The sequence alignment of the amino acids encoding by *StGA2ox1* gene and those of related *GA2ox* genes, such as *SlGA2ox*, *NtGA2ox*, *PnGA2ox*, and *CaGA2ox* genes, was performed. As shown in Fig. 2b, the sequence comparison of *StGA2ox1* with the other GA2-oxidase genes showed that the encoding protein of *StGA2ox1* shared 747.3, 79.3, and 48.5% amino acid identity with the *SlGA2ox1*, *SlGA2ox2*, and *SlGA2ox3*, respectively. The amino acid sequence identity of *StGA2ox1* from *PnGA2ox1* and *PnGA2ox2* was 73.9 and 73.5%, from *NtGA2ox1*, *NtGA2ox2*, and *NtGA2ox3* was 81.5, 81.0, and 85.8%, respectively. And the amino acid identity of *StGA2ox1* was 48.3% from *CaGA2ox*. The highest identity of *StGA2ox1* was observed with *NtGA2ox3*, while the comparatively low identity was *SlGA2ox1*, *SlGA2ox3*, and *Capsicum annuum GA2ox*. The phylogenetic tree of *StGA2ox1* full-length

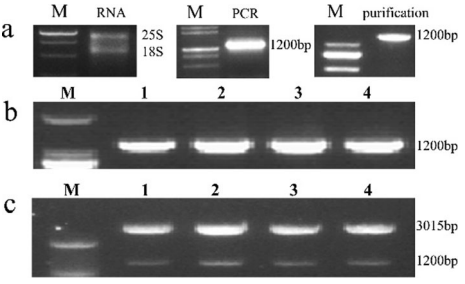
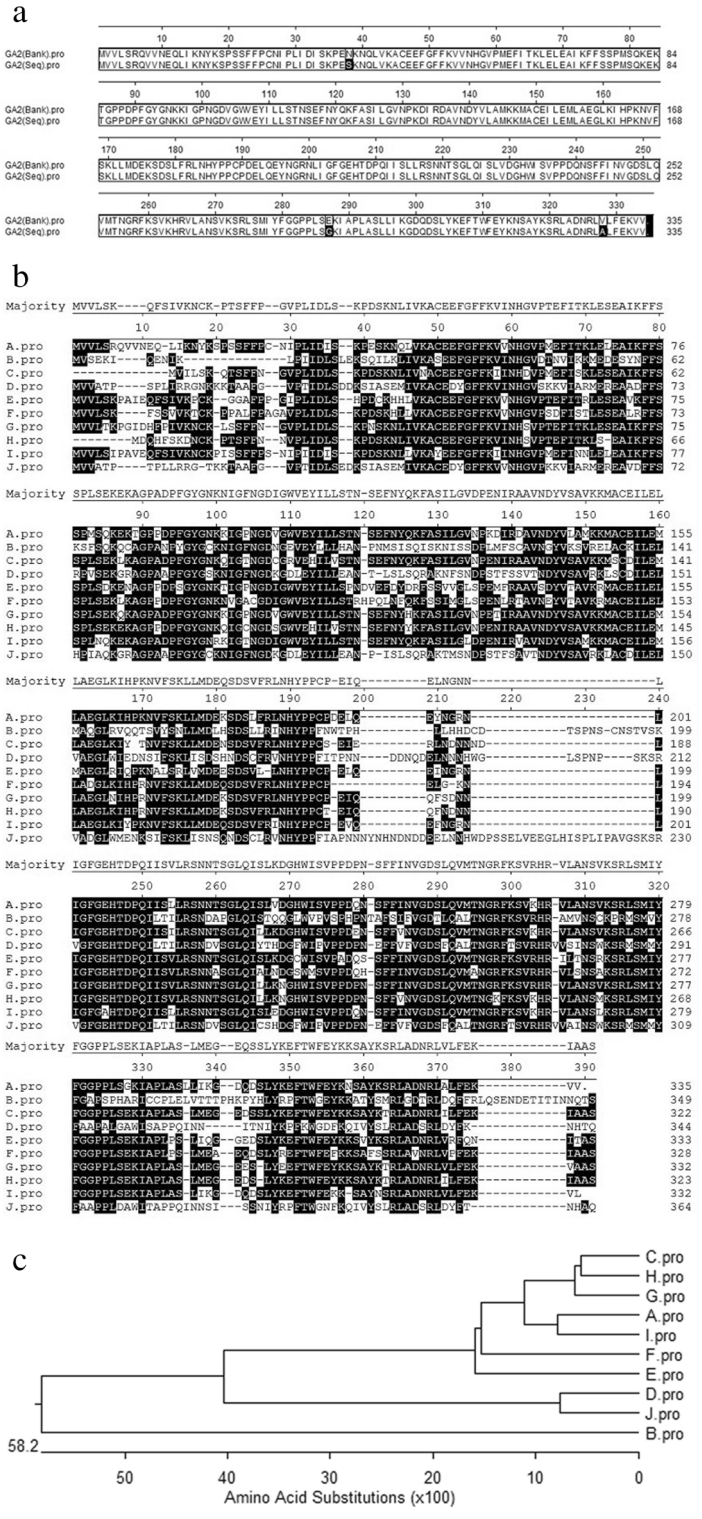


Fig. 1 **a** Extraction of RNA from Qingshu 9 potato, the PCR amplification and product purification of *StGA2ox1*. **b** PCR detection of recombinant vector *StGA2ox1*-T. **c** Enzyme digestion of *StGA2ox1*-T with Sac I and BamH I


a
b
c
C.pro
H.pro
G.pro
A.pro
I.pro
F.pro
E.pro
D.pro
J.pro
B.pro
58.2
50
40
30
20
10
0
Amino Acid Substitutions (x100)

◀ **Fig. 2** **a** Comparison of *GA2ox1* coded amino acid. **b** The homologous alignment of *StGA2ox1* gene. **c** Phylogenetic tree of *StGA2ox1* full-length protein with GA2 oxidases from other plants. The phylogenetic tree was constructed by the MegAlign program. The accession number of each appended protein is as follows: A *StGA2ox1*, B *SlGA2ox1*(NCBI: NM_001247936.2), C *SlGA2ox2* (GenBank: EF017805.1), D *SlGA2ox3* (NCBI: NM_001247818.2), E *PnGA2ox1* (GenBank: GU189414.2), F *PnGA2ox2* (GenBank: GU911364.2), G *NtGA2ox1* (GenBank: AB125232.1), H *NtGA2ox2* (GenBank: AB125233.1), I *NtGA2ox3* (GenBank: EF471117.1), J *CaGA2ox* (GenBank: DQ465393.1)

protein with GA2 oxidases from other plants showed that *StGA2ox1* and *NtGA2ox* can be classified in a group (Fig. 2c).

Construction of pCAEZ1383-*StGA2ox1* Vector

The recombinant plasmid pCAEZ1383-*StGA2ox1* comes from the large fragment of pCAEZ1383 and the small fragment (*GA2ox1* gene) of *GA2ox1*-T plasmid DNA, under the digestion of restriction enzymes Sac I and BamH I. The *StGA2ox1* cDNA was inserted downstream of the CaMV35S in pCAEZ1383. PCR amplification and enzyme digestion were carried out to detect the correctness of recombinant vector. As expected, a PCR product of 1200 bp was obtained, and two enzyme digestion products of 1200 and 11,800 bp were obtained (Fig. 3). That means that the construction of pCAEZ1383-*StGA2ox1* vector was successful. Then, the vector was transformed to competent EHA105 cells, and the target fragment was obtained in bacterial liquid (Fig. 3c).

Analysis of *StGA2ox1* Gene Expression in Transgenic Potato

In order to obtain the transgenic potato plants (M1), the stems and leaves of M1 were co-cultured in MS basal medium with the bacterial liquid of pCAEZ1383-*StGA2ox1* vector. By agrobacterium-mediated transformation as described by Tan [22], the transgenic potato plants were generated (Fig. 4a).

To confirm the presence of *StGA2ox1* transgene in the genome of putative transgenic plants, a PCR analysis was performed using the specific primers of hygromycin resistance gene in pCAEZ1383-*StGA2ox1* vector. As expected, a PCR product of 552 bp was obtained in 7 of 30 plants (Fig. 4b). The presence of hygromycin resistance gene means the presence of pCAEZ1383-*StGA2ox1* vector and means the presence of the *StGA2ox1* transgene.

The qRT-PCR analyses (Fig. 4c) showed that the *StGA2ox1* transcript was overexpressed in the seven positive transgenic lines tested (9, 14, 24, 25, 26, 27, and 29). By comparing with the non-transgenic plant (M1), the *GA2ox1* gene expression of transgenic lines was 2.08, 1.41, 3.05, 1.16, 4.11, 5.02, and 1.35, respectively. The transgenic lines 9, 24, 26, 27's *GA2ox1* gene expression increased significantly. While the up-regulation of *GA2ox1* gene in transgenic lines

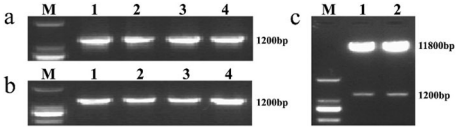


Fig. 3 The construction of pCAEZ1383-StGA2ox1 vector. **a** PCR amplification of the specific StGA2ox1 gene from recombinant plasmid pCAEZ1383-StGA2ox1. **b** PCR amplification of the specific StGA2ox1 gene from the bacteria liquid of agrobacterium EHA105. **c** Digestion of recombinant plasmid pCAEZ1383-StGA2ox1 using Sac I and BamH I

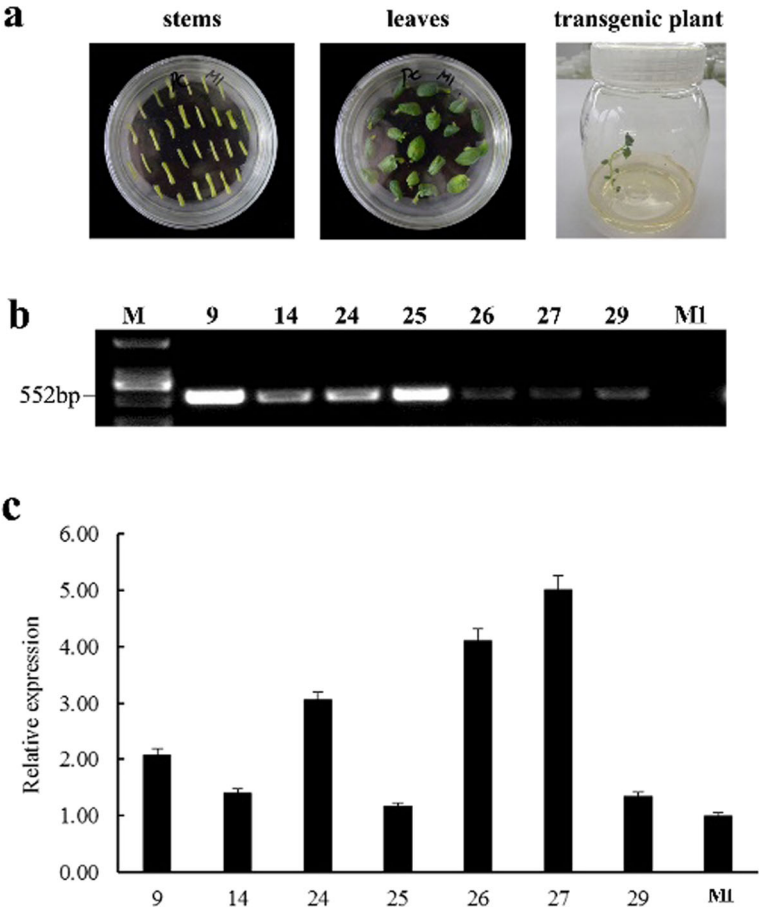


Fig. 4 **a** Transgenic potato plants generated by agrobacterium mediated. **b** PCR amplification of the hygromycin resistance gene from genomic DNA of the transgenic plants. **c** The *StGA2ox1* gene expression of transgenic potato lines and non-transgenic plant (M1)

14, 25, and 29 was smaller than above lines, the difference reached significant level also ($P < 0.05$).

The Response of Transgenic Potato to Abiotic Stresses

In plants, GAs play a regulatory role in development of plant. In order to determine the putative function of *StGA2ox1* in response to abiotic stresses, the gene expression was examined under different stress conditions (200 mM NaCl, 50 μM ABA, 4 °C, 10% PEG). After 48 h of treatment, the expression levels of *StGA2ox1* were measured in different organs, including root, stem, and leaf, to test the tolerance of transgenic plant under salt, hormone, drought, and low-temperature stress. The transgenic line 27(M1–27) was chosen as material in this experiment.

The qRT-PCR analyses shown that (Fig. 5), under the salt stress condition, the *StGA2ox1* mRNA levels of M1–27 were increased in roots, stems, and leaves, compared to M1. The expression quantity of M1–27 is 3.6 times than M1 in roots, 2.1 times than M1 in leaves, the

difference reached an extremely significant level ($P < 0.01$), indicating that the salt stress of 200 mM NaCl affects the *StGA2ox1* expression. Research has shown that ABA plays an important role in regulation of gene expression in response to abiotic stresses [23]. After treatment of 50 μM ABA for 48 h, there was a slight induction of *StGA2ox1* expression that was observed in roots and stems compared to M1, while significantly induced in leaves ($P < 0.01$). PEG treatment showed that there was no accumulation of *StGA2ox1* transcript in roots compared to M1, but a higher expression levels in stems and leaves. The expression quantity of M1–27 is 1.7 times than M1 in stems, 2.5 times than M1 in leaves, the difference reached an extremely significant level ($P < 0.01$). In addition, cold treatment at 4 °C induced *StGA2ox1* expression in all tissues compared to M1, while in stems, a significant induction was observed (2.9 times than M1).

The Expression of Stress Responsive Genes in Transgenic Potato

In plants, the expression of stress responsive genes is used to be a marker to respond to abiotic stress. *RD28* is a member of the dehydration response gene family, and the expression of *RD28* is up-regulated under drought stress conditions [24]. Dehydration-responsive element binding (*DREB*), *WRKY* transcription factors, and function gene *SnRK* play an important role in controlling the expression of abiotic stress responsive genes. At present, *DREB1*, *WRKY1*, and *SnRK2* genes are found to be related to stress resistance in potato [25–27].

To further verify the tolerance of transgenic plants to abiotic stresses, the expression of *RD28*, *DREB1*, *WRKY1*, and *SnRK2* in M-27 and M1 plant under different stress conditions (200 mM NaCl, 50 μM ABA, 4 °C, 10% PEG) was examined. The qRT-PCR analyses show that (Fig. 6),

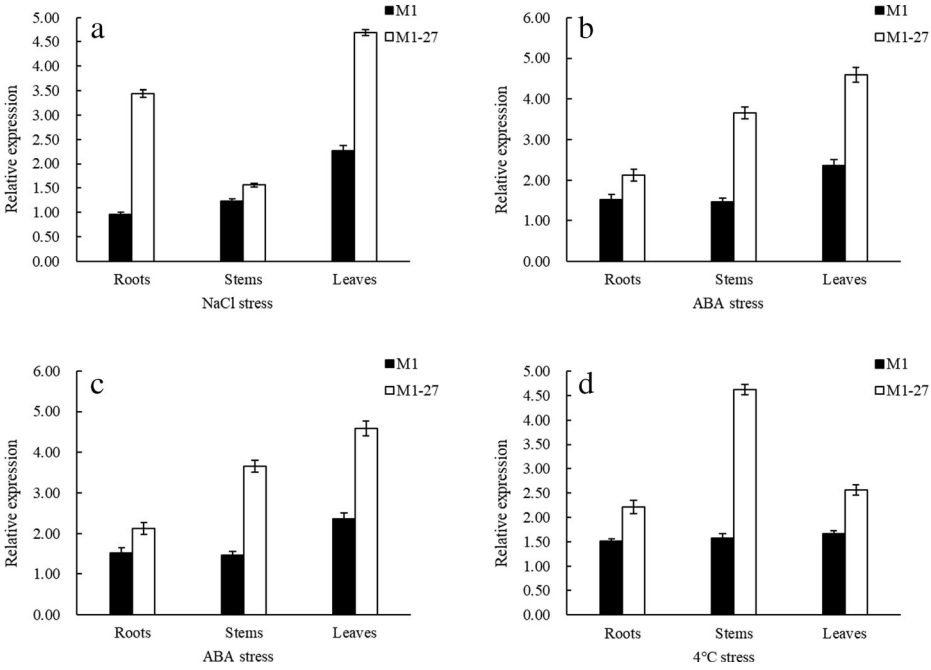


Fig. 5 *StGA2ox1* expression in tissues of potato after 48-h treatment with 200 mM NaCl, 50 μM ABA, 10% PEG, and 4 °C

under the salt and simulating drought stress condition, the *RD28*, *DREB1*, *WRKY1*, and *SnRK2* mRNA levels of M1–27 were significantly increased, compared to M1, and the difference reached an extremely significant level ($P < 0.01$). Among them, the highest *DREB1* gene expression in M1–27 was 4.56 and 4.21 times that of M1, respectively. Under ABA treatment condition, the expression of *DREB1*, *WRKY1*, and *SnRK2* genes is 1.84, 2.23, and 5.51, respectively; the difference reached an extremely significant level ($P < 0.01$). Under cold treatment condition, *RD28*, *DREB1*, and *WRKY1* mRNA levels of M1–27 were significantly increased, compared to M1, and the difference reached an extremely significant level ($P < 0.01$). However, the expression level of *SnRK2* gene was no significant difference from the control.

StGA2ox1 Expression Enhances the Tolerance of Transgenic Plants to Drought

Dehydration treatment was carried out on four transgenic lines (9, 14, 25, 27), and the non-transgenic plant was chosen as control. Three plants of each line were processed. Under the condition of withholding water for 20 days, a significantly difference was observed between the transgenic and M1 plants. In fact, the M1 plants withered while the transgenic plants grew well (Fig. 7).

In order to better understand the response of transgenic plants to drought stress, the chlorophyll, carotenoid, and free proline contents were measured after 20 days of withholding water. As shown in Fig. 7b, a significant decrease of chlorophyll content was observed in M1, while in transgenic plants, this decrease was less important, but still statistical significantly different ($P < 0.01$). Indeed, the total chlorophyll content loss in M1 plants was 61.40%, whereas in transgenic plants of overexpressing *StGA2ox1* gene, the total chlorophyll content

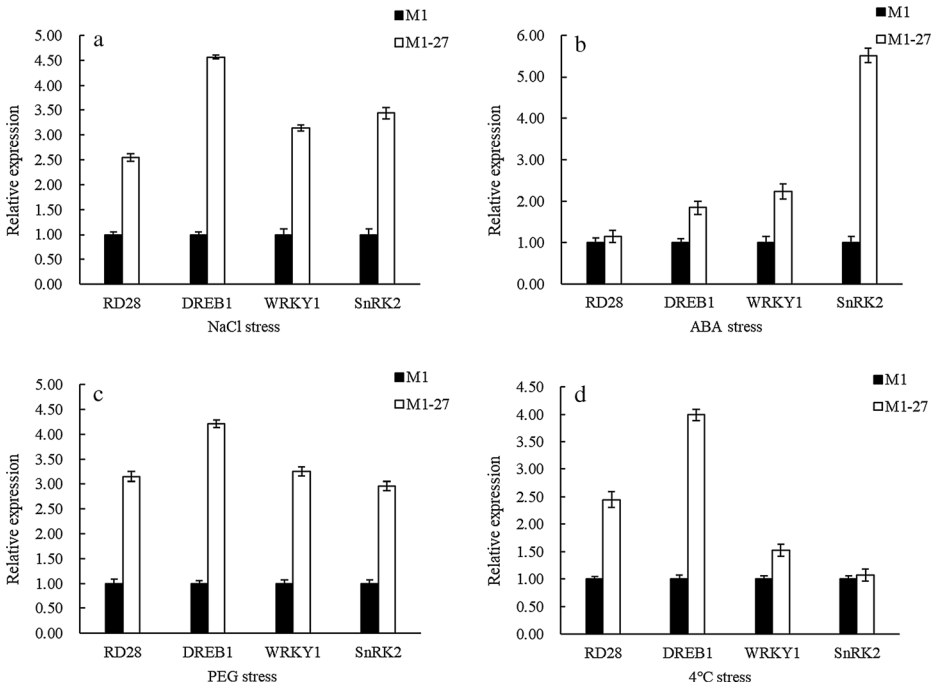


Fig. 6 The expression of marker stress responsive genes in M1–27 and M1 plants under stress conditions

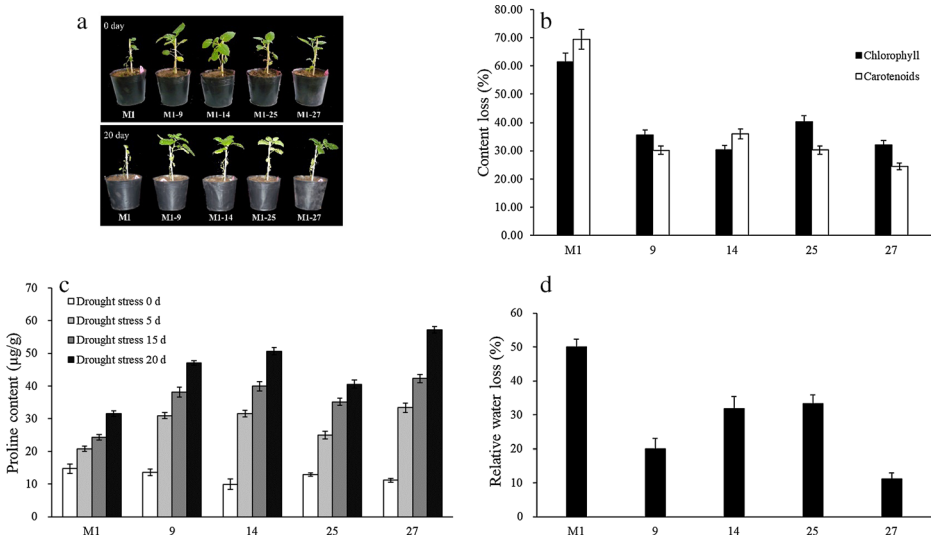


Fig. 7 **a** The growth of transgenic plants under drought stress. **b** The chlorophyll and carotenoid content loss of M1 plant and transgenic plants under the dehydration treatment for 20 days. **c** The free proline content of M1 plant and transgenic plants under the dehydration treatment during 20 days. **d** The relative water content of M1 plant and transgenic plants under the dehydration treatment for 20 days

loss varied from 30.40% in transgenic line 14 to 40.39% in transgenic line 25, and the difference reached an extremely significant level ($P < 0.01$), compared to M1.

The carotenoid content loss was in agreement with chlorophyll. A significant decrease of carotenoid content was observed in M1, while less decrease in transgenic lines. Indeed, the carotenoid content loss in M1 leaves was 69.45%, whereas in transgenic plants of overexpressing *StGA2ox1* gene, the carotenoid content loss ranged between 24.51% in transgenic line 27 and 36.03% in line 14, and the difference reached an extremely significant level ($P < 0.01$), compared to M1.

The result of proline content showed that, under the standard conditions of 0-day dehydration treatment, no significant difference in the proline content was detected in M1 and *StGA2ox1* transgenic lines (Fig. 7c). However, after 5 days of dehydration treatment, the content level of free proline increased in both *StGA2ox1* transgenic plants and M1, progressively, but the increase in transgenic plants was much higher than M1, especially after 20 days of withholding water treatment, and the difference reached an extremely significant level ($P < 0.01$), compared to M1. Indeed, the up-regulation level of proline in transgenic lines was 3.12 to 5.12 times higher than those measured under standard condition of 0-day treatment, whereas the M1 proline content increased 2.13-fold compared to standard condition of 0-day dehydration treatment.

To further examine the osmotic adjustment capacity of transgenic potato plants, the relative water content (RWC) of transgenic lines and M1 was measured after 20 days of dehydration treatment (Fig. 7d). There has a decrease of RWC that occurred in the transgenic lines and M1 plant. Interestingly, a significant decrease in the RWC of 50% was observed in M1, whereas all the transgenic lines exhibited lower decrease of the RWC compared to M1, and the difference reached an extremely significant level ($P < 0.01$). Indeed, the RWC decrease of transgenic lines ranged between 11.1% in line 27 and 33.3% in line 25 suggesting that these plants can control their RWC under dehydration conditions. The results are in agreement with that of chlorophyll

and free proline contents. All these data confirm that overexpression of *StGA2ox1* gene in transgenic potato plants can enhance the tolerance to dehydration compared to M1 plant.

Discussion

In this research, we describe the isolation and characterization of *GA2ox1* gene from potato (Qingshu 9), termed *StGA2ox1*. The sequence of *StGA2ox1* is 1005 bp long and encoding 334 amino acids. In the secondary structure of *GA2ox1* gene encoding protein, there have been 34.43% of alpha helix, 34.13% of random coil, 22.16% of extended strand, and 9.28% of beat turn. This structure is a stable protein since the 33.47 of instability index and the aliphatic index is 88.08. In addition, the most amino acids belong to the hydrophilic amino acids, did not form a larger hydrophobic surface. Alignment analysis and domain comparison suggest that *StGA2ox1* have the highest identity with tobacco *GA2ox3* (*NtGA2ox3*).

Here, the hygromycin resistance gene was chosen as the selection marker gene because it is a downstream element of CaMV35S promoter in binary vector pCAEZ1383. The expression of hygromycin resistance gene represents the expression of recombinant vector pCAEZ1383-*StGA2ox1*. In transgenic lines of overexpression *StGA2ox1* gene, the trait has changed to some extent. In fact, the plant height and leaf area significantly reduced compared to M1 plant. These data are in agreement with previous reports [28–30], which thought that overexpression *GA20ox* gene can promote the gibberellin synthesis and significantly accelerate the growth and development of plant; however, *GA2ox* is the opposite.

Abiotic stress, including salt, dehydration, ABA, and low temperature stress, had some effect on plant growth. The expression of *StGA2ox1* was up-regulated in different tissues. Under salt stress and drought stress, the GA synthesis of plants was inhibited. To cope with adversity stress on plant damage, the expression quantity of *GA2ox* gene increased significantly, *GA20ox* gene expression decreased, to adjust the GA metabolism synthesis. The ABA condition up-regulated the *StGA2ox1* expression in tissues of transgenic plants, for ABA phytohormone involves in several physiologic processes of plant development and increases plant adaptability under different stresses [21]. Achard reported that the decrease of ABA concentration can cause up-regulated expression of *GA2ox* gene and then inhibit the synthesis of gibberellin [19]. On the contrary, exogenous ABA induced the expression of *StGA2ox1* in this research, especially in the leaves, that coincide with the reported results. Low-temperature treatment can improve the expression of *AtGA2ox1* and *AtGA2ox2*, which further inhibit the level of activity of GA to answer adversity stress. In citrus, the expression of *GA2ox* genes was also induced by low temperature [31]. Studies have shown that mild osmotic stress and drought stress can lead to accumulation of GA in *Arabidopsis thaliana* [32]. These data are in agreement with our result that overexpression of *StGA2ox1* in transgenic potato exhibited an enhanced tolerance to low-temperature stress. In addition, the mRNA expression of the reported stresses responsive marker genes (*RD28*, *DREB1*, *WRKY1*, and *SnRK2*) in the transgenic line M-27 and non-transgenic plant were compared under abiotic stress conditions. The up-regulation of *RD28*, *DREB1*, *WRKY1*, and *SnRK2* gene expression in transgenic line M-27 suggesting the enhanced tolerance to abiotic stress.

The changes of plant physiological indexes reflect the tolerance to drought stress. In this article, a significant decrease of chlorophyll, carotenoid, and relative water content was observed in transgenic plant and M1 plant; indeed, the loss of chlorophyll, carotenoid, and relative water in transgenic plants was more than that in M1 plant. Meanwhile, a higher level of proline content was observed in transgenic plants.

Under dehydration condition, synthesis rate of active GA in transgenic plants was decreased but still higher than that in control plant due to the excessive expression of *GA2ox1*. The biosynthesis of gibberellin is closely related to the chlorophyll, carotenoid, and leaf relative water content of plant; in fact, the loss ratio of chlorophyll, carotenoid, and leaf relative water content in transgenic plants was less than control plant. Thus, inducing the higher level of proline content was observed in transgenic plants. These data suggest that the transgenic plants of overexpression *StGA2ox1* showed increased tolerance to drought stress.

Conclusion

In this article, the *StGA2ox1* gene of potato Qingshu 9 was isolated by homology cloning. The full length of cDNA is 1005 bp, and the cDNA corresponds to a protein of 334 amino acids. The molecular weight of *StGA2ox1* encoding amino acids is 37,687.48 Da, and the isoelectric point is 8.324. The amino acids encoding by *StGA2ox1* have the highest identity with *NtGA2ox3*. Here, a binary vector pCAEZ1383-*StGA2ox1* was constructed. And the *StGA2ox1* was overexpressed in transgenic plants. Overexpression of *StGA2ox1* in transgenic potato exhibited an enhanced tolerance to salt, dehydration, low-temperature stress, and ABA treatment, compared to non-transgenic plant. That result was supported by data of the up-regulation of *RD28*, *DREB1*, *WRKY1*, and *SnRK2* gene expression in transgenic line M-27, higher proline content, smaller chlorophyll and carotenoid content loss, and relative water content loss in transgenic plants of *StGA2ox1* overexpression. All these data suggest that *StGA2ox1* play an important role in synthesis pathway of gibberellic acid and the regulation of potato growth and development.

Materials and Methods

Plant Materials

Two potato plants (*Solanum. tuberosum* L.) cultivars, Qingshu 9 (Q9) and Mingshu 1 (M1), were used in this study. Potato plants were cultivated in vitro and propagated in solid MS basal medium in a growth chamber at 25 ± 2 °C for 12/12-h photoperiod, use supplementary lighting during the day [33].

Isolation and Cloning of *StGA2ox1* cDNA

Total RNA was isolated from 3-week-old plants of Q9 in vitro cultivated, followed the instructions of plant total RNA extraction kit (Tiangen). And the first chain cDNA of potato genome was synthesized from total RNA as described by "FastQuant RT Kit" (Tiangen).

The full coding *GA2ox1* sequence (CDS) was found in the National Center for Biotechnology Information (NCBI) website and amplified by PCR using specific primers (forward primer 5′-ACGCGAGCTCCAAATCTCTTTAATTTCCACA-3′ and reverse primer 5′-ACATGGATCCAACTCGTAACGAGACTTCATA-3′), the forward primer contains a Sac I site (GAGCTC) and reverse primer contains a BamH I site (GGATCC) to facilitate the construction of plant expression vector. After recycling and purification, the PCR product was inserted in pGEM®-T easy vector (Promega) by T4 ligase. The ligation reaction was carried out by adding 1 μL of

pGEM®-T easy vector, 1 μL of PCR product, 1 μL of T4 DNA ligase, and 5 μL of 2× rapid ligation buffer in 10 μL of final volume reaction mixture. The reaction was mixed by pipetting and incubated at room temperature for 1 h. Five microliters of the reaction mix was used to transform 50 μL of competent DH5α cells. A PCR with specific primers and an enzyme digestion reaction with Sac I and BamH I was carried out to detect the presence of the insert.

The DNA sequencing was carried out on bacterial colony with correct insert. Using the NCBI BLAST search program, the database searches were performed. Alignment of the potato *GA2ox1* protein with other related plant *GA2ox1* protein was performed by means of the MegAlign multiple alignment program of DNAstar7.1 Version software. Phylogenetic analysis was performed by the UPGMA method [34] with the aid of DNAstar7.1 Version software.

Construction of Plant Expression Vector

The binary vector pCAEZ1383 was combined from vector pCAMBIA1341 and pEZR(K)-LC, pCAMBIA1341 has the selective marker gene hygromycin gene (Hyg), and pEZR(K)-LC has the multiple cloning site needed in this study. After enzyme digestion reaction with Sac I and BamH I on pCAEZ1383 and pGEM®-T easy vector, fragment recycling reaction with "TIANgel Midi Purification Kit" (Tiangen), ligation reaction with T4 ligase (Promega), the *GA2ox1* gene was transferred into the vector pCAEZ1383. These reactions were carried out by following the instructions and followed by the transformation and screening of *Escherichia coli* DH5α strain. A PCR with specific primers and an enzyme digestion reaction with Sac I and BamH I was carried out to detect the presence of the insert.

Generation of Transgenic *StGA2ox1* Potato Plants

Solanum tuberosum L. M1 cultivar was used to produce transgenic plants. The recombinant vector pCAEZ1383-*StGA2ox1* was transferred in Agrobacterium tumefaciens EHA105 that was subsequently used for stem and leaf transformation. Potato transformation was performed as described by Bouaziz [35]. After obtaining the transgenic plants, these putative plants were multiplied in MS basal medium [33] containing 6 mg/L hygromycin and 300 μg/L cefotaxime sodium salt at 25 °C.

Identification of Putative Transgenic Plants

The genomic DNA of putative positive potato plants was extracted from tissue culture seedling as described by Dellaport [36] and was used in PCR-based identification of transgenic plants. To detect positive lines, a couple of primers (forward primer 5′-GCCGATCTTAGCCA GACGAG-3′ and reverse primer 5′-TTGTGTACGCCCGACAGTCC-3′) of hygromycin resistance gene in vector pCAEZ1383-*StGA2ox1* were used.

The PCR was performed in a final volume of 20 μL containing 30 ng of genomic DNA, 2 μL of 2× buffer, 1.8 μL of dNTPs (2.5 mM each), 0.5 μL of each hygromycin resistance gene primers (10 μM), and 0.4 U of Taq DNA polymerase (Tiangen). The amplified product was resolved on a 0.8% agarose gel and visualized by ethidium bromide staining [21].

All the transgenic lines that had been identified correctly were selected to analyze the expression of *StGA2ox1* gene. Total RNA of transgenic lines was extracted by using the "RNAprep Pure Plant Kit" (Tiangen), and the cDNA was synthesized by means of the "FastQuant RT Kit" (Tiangen). The *StGA2ox1* gene expression in transgenic lines was analyzed

by quantitative real-time PCR (qRT-PCR) using the *StGA2ox1* specific primers (forward primer 5′-ATCACAACAAATCCATCA-3′ and reverse primer 5′-AGCACCATACATCCCATA-3′). For Actin was often used as the housekeeping gene in expression analysis for its stabilization [37–40], so the Actin gene (Gene Bank: GQ339765.1) was used as reference gene and the sequences of its specific primers are presented here (forward primer 5′-AACAAGAGGACAAGGCTGCC-3′ and reverse primer 5′-TCCAAGAATAACCCCGACGA-3′).

Quantitative Real-Time PCR Analyses

The qRT-PCR analyses of transgenic line were performed to investigate in response to exogenous salt, ABA, cold, and drought stress treatments. These treatments were performed on 3-week-old tissue culture seedling. For salt stress treatment, the transgenic line and non-transgenic plant were transferred in solid MS basal medium containing 200 mM NaCl. For ABA stress treatment, they were transferred in solid MS basal medium containing 50 μM ABA [21]. For drought stress treatments, they were transferred in solid MS basal medium supplemented with 10% (*w*/*v*) polyethylene glycol with an average molecular weight of 6000 (PEG 6000). For cold stress treatment, the plants were transferred solid MS basal medium in a growth chamber at 4 °C for 12/12-h photoperiod. All stress treatments were performed for 48 h.

Total RNA was isolated, from root, stem, and leaf of stress treatment potato plant, for the analysis of *StGA2ox1* gene expression. The RNA concentration and quality were measured by nucleic acid analyzer (Bio-Rad, US). The expression of *StGA2ox1* was performed based on qRT-PCR and was investigated in transgenic lines and M1 potato plants using specific primers (GA2ox1: forward primer 5′-ATCACAACAAATCCATCA-3′ and reverse primer 5′-AGCA CCATACATCCCATA-3′, Actin: forward primer 5′-AACAAGAGGACAAGGCTGCC-3′ and reverse primer 5′-TCCAAGAATAACCCCGACGA-3′). The Actin gene was performed under the same conditions to normalize the amount of template added. One microliter of each cDNA was used for PCR, and the reaction was carried out by adding 10 μL of 2 × SYBR Green I Mix, 1 μL of each specific primer (10 μM) in 20 μL of reaction mixture. The reactions were performed at 95 °C for 5 min, followed by 40 cycles of 95 °C for 10 s and 60 °C for 30 s, and then collect the fluorescent. The temperature of melting curve was increased from 65 to 95 °C by 0.2 °C per second. The data were analyzed with the iQ™ 5 Optical System Software Version 2.1 (Bio-Rad, US).

Greenhouse Planting

Three-week-old in vitro cultivated plants from transgenic lines and non-transgenic that had developed roots in MS basic medium were transferred to flower pot in greenhouse under the temperature conditions of 25 ± 2 °C. One week later, the length above ground and the leaf acreage of transgenic plants were measured, and drought stress was carried out by withholding water for 20 days.

Measurement of Chlorophyll Content, Free Proline, and Relative Water Content

After being subjected to drought stress, the healthy and full expanded leaves from transgenic lines and non-transgenic were collected every 5 days to measure the chlorophyll content and free proline [41, 42].

The relative water content (RWC) of the transgenic plants and M1 was detected as described by Yamasaki and Dillenburg [43]. After drought stress for 10 days, the leaves of transgenic plants and M1 were cut from tissue culture seedling. Their fresh weight (FW) was measured first, and then, the leaves were floated in deionized water for 7 h to determine the turgid weight (TW). Finally, the leaves were dried in 80 °C for 48 h to obtain the dry weight (DW). The RWC was then calculated by the following formula: RWC (%) = [(FW-DW) / (TW-DW)] × 100.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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