14-3-3 gene of Zostera japonica ZjGRF1 participates in gibberellin signaling pathway

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Abstract

Background Zostera japonica is a unique seagrass species in Asia with important ecological value. Gibberellin (GA) is a plant hormone crucial in the regulation of plant growth and development, including seed longevity, seed germination, anti-aging, hypocotyl elongation, leaf development, reproductive organ development, and abiotic stress responses. However, the role of 14-3-3 gene of Z. japonica in GA signaling pathway remains unclear.

Methods and results Herein, gibberellin content and expression of GA synthesis genes were lower in Arabidopsis overexpressing ZjGRF1, 14-3-3 gene of Z. japonica, than in wild type (WT). Moreover, the expression level of GA receptors was lower in transgenic ZjGRF1 Arabidopsis than in WT. The expression level of GA response gene expansin8 (EXP8) was lower in transgenic ZjGRF1 Arabidopsis than in WT. In contrast, the expression levels of PACLOBUTRAZOL RESISTANT1 (PRE1), PRE3 and SCARECROW-LIKE 3 (SCL3) were higher in the transgenic ZjGRF1 Arabidopsis than in WT. Transgenic ZjGRF1 Arabidopsis has decreased sensitivity to paclobutrazol, an inhibitor of GA synthesis. The expression level of flowering regulatory genes was lower in transgenic ZjGRF1 Arabidopsis than in WT.

Conclusion This is the first study to report the function of 14-3-3 gene family in seagrass. These findings can be used in future studies on the regulation role of 14-3-3 gene on plant development.

Keywords 14-3-3 · Zostera japonica · ZjGRF1 · Gibberellin · Arabidopsis thaliana

Introduction

Gibberellins (GAs) are growth factors widely found in plants. They are diterpenes consisting of four isoprene units. Although 136 GAs with different structures have been isolated and identified from plants, fungi, and bacteria, only a few have biological activities. GA1 and GA4 are the main endogenous active molecules in most plants. Most GAs are precursors or inactive metabolites of active GAs. The concentration of active GAs in plant tissue is determined by the GAs synthesis and inactivation rate. GA regulates many aspects of plant growth and development by promoting cell proliferation and expansion, including flower and fruit development, seed germination, stem extension, leaf expansion, etc.

GA synthesis in higher plants is mainly divided into three stages, catalyzed in plastid, endoplasmic reticulum and cytoplasm. In plastids, copalyl diphosphate synthase (CPS) and kaurene synthase (KS) catalyze the precursor of GA, geranylgeranyl pyrophosphate (GGPP), to form kaurene. In the endoplasmic reticulum, kaurene oxidase (KO) and kaurenoic acid oxidase (KAO) catalyze kaurene to form GA12. In the cytoplasm, GA3 oxidase (GA3ox) and GA20 oxidase (GA20ox) catalyze GA12 to form GA1 and GA4 with high biological activity [1].

As a maize GA-deficient mutant, d1 has short plant and monogamous flower phenotype. It encodes GA3ox2 in maize [2]. D1 can also catalyze GA20 to form GA1 and GA4, GA3 to form GA3, and GA9 to form GA9 in vitro. GA3ox1 encoded by GNP1, can also catalyze GA synthesis [3]. GAS2 in Arabidopsis can catalyze GA12 to from an atypical GA DHGA12 with high biological activity [4].
GA has a similar effect as other plant hormones. Too much or little GA significantly impacts the growth and development of plants. Therefore, plants regulate GA synthesis strictly. GAMYBL2 directly inhibits the expression of CPS and GA3ox2 in rice [5]. Brassinosterol inhibits the synthesis of GA in vivo by inhibiting rice miR159D and activating the target gene GAMYBL2 of miR159D.

The 14-3-3 proteins (GENERAL REGULATORY FACTOR, GRF) were first found in bovine brain tissue cells [6], and were named according to the number of fragments separated by DEAE cellulose and the mobility in starch gel electrophoresis. Brain tissue is rich in 14-3-3 proteins, accounting for about 1% of all soluble proteins. For a long time, 14-3-3 protein is considered to be a brain specific protein. Later, it was found that it is a highly conserved regulatory protein widely existing in eukaryotes. Various 14-3-3 proteins have been found in plants since 1992. For instance, 13 genes encoding 14-3-3 protein have been identified in Arabidopsis. The 14-3-3 proteins interact with target proteins by recognizing specific phosphorylation sequences. More than 300 target proteins can interact with 14-3-3 proteins in plants. Various 14-3-3 isoforms are expressed in different plant tissues. These 14-3-3 isoforms specifically regulate target proteins and participate in various signal transduction and metabolic processes in plant cells, thus influencing the growth and development of plants.

REPRESSION OF SHOOT GROWTH (RSG) is a bZIP transcription factor, which regulates GA levels in plants by regulating the transcription of related genes in the GA synthesis pathway. For instance, 14-3-3 protein in tobacco interacts with RSG at RSG-Ser-114. The interaction between 14-3-3 protein and RSG depends on Ser-114 phosphorylation of RSG. Exogenous application of GA temporarily increases intracellular Ca\(^{2+}\) level, making Ca\(^{2+}\)-dependent CDPK kinase phosphorylate Ser-114 of RSG, thus promoting the interaction between 14-3-3 protein and the phosphorylated RSG. In this process, the 14-3-3 protein, as a negative regulator, binds to RSG, thus changing the localization of RSG protein in cells, making RSG remain in the cytoplasm. As a result, RSG cannot enter the nucleus to regulate the genes encoding GA biosynthesis-related proteins and inhibit the transcription of GA biosynthesis-related genes in the nucleus, thus controlling the GA level in vivo [7, 8].

The Seagrass meadow ecosystem is a highly productive ecosystem in coastal waters [9]. Despite accounting for only 0.15% of the global ocean area, it contributes 1% of the net primary productivity of the global ocean. The ecological function and economic value of seagrass meadows have been gradually recognized in recent years [10].

Zostera japonica is a Subgenus Zostera species endemic in Asia. It is short, and has important ecological value. Z. japonica can live in the shallow intertidal zone, where the water temperature and light fluctuation are strong. However, Zostera marina cannot adapt to such environment, showing that Z. japonica has stronger adaptability and vitality [11]. Z. japonica is the most widely distributed seagrass species in China. It is distributed in the Yellow Sea, Bohai Sea, South China Sea, and other coastal areas in China. Z. japonica in Weifang, Qingdao, Yantai, Weihai, Rizhao, and other places in Shandong Province are found in the Yellow Sea and Bohai Sea areas [12]. However, the seagrass habitat in the Yellow Sea and Bohai Sea has been seriously damaged in recent years. Moreover, finding a large area with a continuous distribution of Z. japonica seagrass meadows is difficult.

To the best of our knowledge, no study has assessed the physiological function of the seagrass 14-3-3 gene. In this study, a 14-3-3 gene ZjGRF1 of Z. japonica was cloned and overexpressed in Arabidopsis with 35 S promoter to obtain homozygous plants. GA content and expression of GA synthesis genes were lower in transgenic ZjGRF1 Arabidopsis than in wild type (WT). The expression level of GA receptors and GA response gene expansin8 (EXP8) was lower in transgenic ZjGRF1 Arabidopsis than in WT. Moreover, the expression levels of PACLOBUTRAZOL RESISTANT1 (PRE1), PRE5, and SCARECROW-LIKE 3 (SCL3) were higher in transgenic ZjGRF1 Arabidopsis than in WT. Transgenic ZjGRF1 Arabidopsis was less sensitive to paclobutrazol, an inhibitor of GA synthesis. The expression level of flowering regulatory genes was lower in transgenic ZjGRF1 Arabidopsis than in WT. In conclusion, these results suggest that ZjGRF1 is involved in GA signaling pathway.

**Materials and methods**

**Plant materials and growth conditions**

In this study, Arabidopsis Columbia ecotype was used as WT. Seedlings (17-day-old) were grown on 1/2 Murashige and Skoog (1/2 MS) solid medium (0.8% agar) in a growth chamber (ZMD-260, Yanghui, Ningbo, China) under long-day conditions, at 16 h of white light (80 µmol m\(^{-2}\) s\(^{-1}\)) and 8 h of darkness, 60% relative air humidity, and 21 °C.

**Isolation of ZjGRF1 from Z. japonica**

An RNAprep Pure Plant Kit (DP441, Tiangen, Beijing, China) was used to isolate total RNA from Z. japonica leaves following the manufacturer's instructions. The RNA was reverse-transcribed using PrimeScript™ RT reagent Kit with gDNA Eraser (RR047A, Takara, Japan). The primers ZjGRF1-pGWB17-F and ZjGRF1-pGWB17-R were amplified using first-strand cDNA as a template (Table S1). PCR
was conducted using 2× TransStart® FastPfu PCR SuperMix (-dye) (AP221-01, Tran, China) as follows: preheating at 95 °C for 5 min, then 40 cycles of denaturation at 95 °C for 1 min, annealing at 50 °C for 20 s and extension at 72 °C for 30 s, then a final extension at 72 °C for 5 min. The product was cloned into TOPO® vector (K240020, ThermoFisher, USA), following the manufacturer’s instructions, then sequenced. The 35 S:ZjGRF1 construct was generated by cloning the fragment in the pGWB17 binary vector using Gateway® BP Clonase™ II Enzyme Mix (11,789–020, Invitrogen, USA), following the manufacturer’s instructions.

Plasmid Construction and Plant Transformation

The 35 S: ZjGRF1 recombinant vector was introduced into Agrobacterium tumefaciens C58 via freeze-thaw method. A. thaliana (Col-0) was transformed with a single positive colony. Transgenic Arabidopsis plants were obtained via A. tumefaciens-mediated floral dipping. Further experiments were performed using homozygous transgenic plants of T3 generation.

qRT-PCR

Quantitative real-time PCR was performed with TB Green Premix Ex Taq II (Takara) using QuantStudio 3 real-time PCR detection system (applied biosystems by Thermo Fisher Scientific). RNAprep pure plant Kit (Tiangen) was used to isolate total RNA from frozen samples in liquid nitrogen, following the manufacturer’s protocol. Prime-script RT reagent kit with gDNA eraser (Takara) was used as internal reference. The relative transcription level was determined via comparative threshold cycle (Ct). The primer name and sequence are shown in Table S1. Each genotype had three independent biological replicates.

GA content measurement

The GA content was measured in the seedlings (17-day-old) using a Plant GA ELISA Kit (Senbeijia, Nanjing, China).

Statistical analysis

Data are presented as means ± SD. Asterisks indicate a significant difference between WT and transgenic ZjGRF1 Arabidopsis using a Student’s t-test (**P < 0.01). Microsoft Excel for Windows (Microsoft office 365, Redmond, WA, USA) was used for all statistical analyses. Three independent biological replicates of each genotype were statistically analyzed for qRT-PCR. Eighteen seedlings were statistically analyzed for measurement of the root length. Sixteen seedlings were statistically analyzed for measurement of the hypocotyl length.

Results

Identification of Overexpressed Plants

Transgenic Arabidopsis lines carrying 35 S: ZjGRF1 construct were constructed to evaluate the function of ZjGRF1 in plants. Three independent transgenic lines were identified by hygromycin resistance. T3 homozygous progenies of transgenic lines (OE1, OE2, and OE3) were selected for further study. RT-PCR was used to detect the transcript level of ZjGRF1 (Fig. S1).

ZjGRF1 inhibits GA synthesis

GA is one of the essential plant hormones in plant growth and development. It regulates seed germination, stem elongation, flower transformation, flowering, and fruit development. Herein, GA content was lower in transgenic ZjGRF1 Arabidopsis plants than in WT (Fig. 1a). Moreover, the expression level of GA3ox1 and GA3ox2 was significantly lower in transgenic ZjGRF1 Arabidopsis plants than in WT plants (Fig. 1b).

The expression level of GA receptors in transgenic ZjGRF1 Arabidopsis and WT

GA signaling pathway has been extensively studied in rice [13] and Arabidopsis [14]. For instance, in rice [13] and Arabidopsis, DELLA protein is the key negative regulator of plant growth and development [15, 16]. GIBBERELLIN-INSSENSITIVE DWARF 1 (GID1) are GA receptors [17],...
which can change their conformation after recognizing and binding to GA and DELLA protein to form GA-GID1-DELLA trimer. GIDI degrades DELLA protein through SCF<sup>GID2</sup>/SLY1 complex, relieves its inhibition on plant growth, and promotes GA effect in plants [18–20]. Herein, the results showed that the expression level of GID1a, GID1b, and GID1c was significantly lower in transgenic ZjGRF1 Arabidopsis plants than in WT plants (Fig. 2).

**ZjGRF1 affects the expression level of GA response-related genes**

In this study, the expression level of EXP8 was significantly lower in transgenic ZjGRF1 Arabidopsis plants than in WT plants. In contrast, the expression levels of PRE1, PRE5, and SCL3 were significantly higher in transgenic ZjGRF1 Arabidopsis plants than in WT plants (Fig. 3).

**The sensitivity of transgenic ZjGRF1 Arabidopsis to Paclobutrazol (PAC)**

In this study, transgenic ZjGRF1 Arabidopsis was less sensitive to GA synthesis inhibitor PAC (Fig. 4). For instance, the root length of WT and transgenic ZjGRF1 Arabidopsis plants decreased by 65% and 53%, respectively, at 1 µM PAC (Fig. 4b). The seed germination rate of transgenic ZjGRF1 Arabidopsis was then studied under white light at 40 µM PAC. WT seeds did not germinate in the presence of 40 µM PAC, while 18% of transgenic ZjGRF1 Arabidopsis seeds germinated (Fig. 4c). The length of hypocotyl was then measured in the presence of 1 µM PAC to further investigate the PAC effect. Seedlings were grown in 1/2 MS medium containing 1 µM PAC in the dark for 12 days. Hypocotyl length decreased from 2.8 cm to 0.7 cm for WT plants and from 2.9 cm to 1.0 cm for transgenic ZjGRF1 Arabidopsis plants when PAC concentration was increased from 0 to 1 µM (Fig. 4d).

**The expression level of flowering regulatory genes in transgenic ZjGRF1 Arabidopsis and WT plants**

ZjGRF1 inhibited flowering under long-day (LD) condition (Fig. 5a and c). Under the LD condition, wild-type plants bolted at 28 d after sowing and showed 15 rosette leaves and two cauline leaves. In contrast, the transgenic ZjGRF1 Arabidopsis bolted at 30–32 d with 17–19 rosette leaves and three cauline leaves. Taken together, these results indicate that ZjGRF1 inhibits flowering under LD condition.

GA promotes flowering by activating the expression of LEAFY (LFY) and SUPPRESSOR OF CONSTANS1 (SOC1) [21, 22]. In this study, the mRNA expression levels of LFY and SOC1 in samples collected 12 h after illumination under LD conditions were determined. The mRNA expression levels of SOC1 and LFY were lower in transgenic ZjGRF1 Arabidopsis than in WT (Fig. 5d). These results suggest that ZjGRF1 inhibits flowering by inhibiting the mRNA expression of LFY and SOC1.

**Discussion**

In tobacco, 14-3-3 protein interacts with RSG at RSG-Ser-114. The interaction between 14-3-3 protein and RSG depends on Ser-114 phosphorylation of RSG. Exogenous application of GA temporarily increases intracellular Ca<sup>2+</sup> level, making Ca<sup>2+</sup>-dependent CDPK kinase phosphorylate Ser-114 of RSG, thus promoting the interaction between 14-3-3 protein and the phosphorylated RSG. In this process, 14-3-3 protein, as a negative regulator, binds to RSG,
GA has a similar effect as other plant hormones. Too much or little GA can significantly impact the growth and development of plants. Therefore, plants regulate GA synthesis strictly. GAMYBL2 directly inhibits the expression of CPS and GA3ox2 in rice [5]. Brassinosterol inhibits the synthesis of GA in vivo by inhibiting rice miR159D and activating the target gene GAMYBL2 of miR159D. SHB encodes AP2/ERF transcription factor, which can directly promote the expression of KS1 [23]. Rice mutant shb has the phenotype of shorter and fewer cortical cells in root tip meristem due to the decreased GA synthesis. GDD1 has transcriptional activity in rice [24]. GDD1 can also directly inhibit the expression of KO2. Deletion of GDD1 shortens rice roots, stems, panicles, and seeds. NAC2 inhibits the expression of KO2 in rice [25]. NAC2 also regulates the plant height and flowering time of rice by regulating GA synthesis. The complex LOL1/bZIP58 can promote the expression of KO2 in rice [26]. Increased GA synthesis and bZIP58-mediated programmed cell death in aleurone layer jointly promote rice seed germination. P2 protein of rice dwarf virus can inhibit the activity of KO2 protein and reduce GA synthesis in plants [27]. Abscisic acid and ethylene induce high expression of HB1 in rose [28]. HB1 can changing the localization of RSG protein in cells, making RSG remain in the cytoplasm. As a result, RSG cannot enter the nucleus to regulate the genes encoding GA biosynthesis-related proteins, inhibit the transcription of GA biosynthesis-related genes in the nucleus, and thus controlling the GA level in vivo [7, 8]. To the best of our knowledge, no study has assessed the physiological function of the seagrass 14-3-3 gene in GA signal pathway. The amino acid identity between ZjGRF1 and Arabidopsis thaliana general regulatory factor 7 is 90%.

In this article, GA content and expression of GA synthesis genes were lower in transgenic ZjGRF1 Arabidopsis than in WT (Fig. 1). The expression levels of GA receptors (Fig. 2) and GA response gene EXP8 (Fig. 3) were lower in transgenic ZjGRF1 Arabidopsis than in WT. However, the expression levels of GA response genes PRE1, PRE5, and SCL3 were higher in transgenic ZjGRF1 Arabidopsis than in WT (Fig. 3). Moreover, transgenic ZjGRF1 Arabidopsis was less sensitive to paclobutrazol, an inhibitor of GA synthesis (Fig. 4). The expression level of flowering regulatory genes was lower in transgenic ZjGRF1 Arabidopsis than in WT (Fig. 5). These results indicate that ZjGRF1 is involved in the GA signaling pathway.

Fig. 4 Transgenic ZjGRF1 Arabidopsis is less sensitive to GA synthesis inhibitor PAC. (a) Graphical representation of the seedlings treated with PAC (2 µM). Seedlings were transferred at day 4 and imaged 10 d later. (b) Root elongation of transgenic ZjGRF1 Arabidopsis grown under the LD condition in the absence or presence of 1 µM PAC for 14 d (SD, n = 18). (c) Increased seed germination in the transgenic ZjGRF1 Arabidopsis in the presence of 40 µM PAC. Imbibed seeds were germinated under white light with or without 40 µM PAC (SD, n = 3). (d) Hypocotyl elongation of transgenic ZjGRF1 Arabidopsis grown in the dark in the presence of 1 µM PAC for 12 d. (SD, n = 3). ** P < 0.01
inhibit the expression of GA20ox1, the GA synthesis gene, and accelerate the senescence of rose. YAB1 inhibits the expression of GA20ox2 in rice [29]. Activation of GA signal can induce YAB1 expression. In contrast, inhibition of GA signal reduces YAB1 expression, suggesting that YAB1 is involved in the feedback regulation of GA signal. RVE1 and RVE2 in Arabidopsis can inhibit the expression of GA20ox2 [30]. brassinosterol promotes GA3ox2 expression in rice through BZR1 [31]. Low concentration of brassinosterol can activate GA3ox2 expression and promote GA synthesis. In contrast, high concentration of brassinosterol can activate GA2ox3 expression and promote the metabolic inactivation of GA. Additionally, GSR, EATB, GD1, WRKY70, and miR396D in rice, miR9678 in wheat, BBX24 in chrysanthemum, ABI4 in Arabidopsis, and phosphorus signals can also affect GA synthesis [32, 33].

The GA content in plants is determined by the relative strength of GA synthesis and decomposition, which is regulated by various key enzymes. Many enzymes are involved in GA synthesis and decomposition in higher plants. GA20-oxidase and GA3-oxidase, as the key enzymes in the last step of GA biosynthesis pathway, can catalyze GA12 and GA33 to form active GA1 and GA4 [34, 35]. GA20ox converts GA12 and GA33 into GA9 and GA20. GA3ox further converts GA9 and GA20 into GA1 and GA4 [36]. Moreover, the negative feedback of active GA regulates GA20ox and GA3ox [37, 38]. GA2–oxidase is crucial in GA degradation. It can decompose and inactivate bioactive GAs and their precursors and other intermediates in plants, thus maintaining the balance between bioactive GAs and intermediates [39]. These three GA oxidase genes belong to the 2OG-Fe (II) oxygenase subfamily and are encoded by the polygenic family [34]. The amount of bioactive GA synthesized is reduced in the absence of GA20ox and GA3ox. Similarly, overexpression of GA2ox gene degrades bioactive GA in plants, resulting in dwarf phenotype [35, 40]. In addition, many studies have found that GA2ox, GA3ox and GA20ox family members have certain effects on flowering through overexpression or mutation [41–44]. GA biosynthesis genes exist in all ferns and flowering plants. Herein, GA content was lower in transgenic ZjGRF1 Arabidopsis than WT, possibly due to the lower expression levels of GA3ox1 and GA3ox2 (Fig. 1).
GID1 proteins are soluble GA receptors. GID1 genes encode proteins similar to Hormone-sensitive lipases (HSL). Although GID1s contain conserved HSL motifs (HGG and GXSXG), they do not have hydrolase activity. GID1s can directly interact with DELLA of rice, which depends on the presence of active GA [17]. AAtGID1s decrease within 3 h after GA4 treatment. However, in Arabidopsis seeds, the expression of AAtGID1s gradually increases from 24 to 48 h of increased GA4 treatment time, possibly due to the difference between short-term and long-term effects of GA. These results indicate that GA4 inhibits GID1 expression in a short time, while it stimulates GID1 expression in a long time [45]. The down-regulation of GID1s in transgenic ZjGRF1 Arabidopsis may be due to the low GA content (Fig. 2).

PREs are GA-responsive genes. PRE gene family consists of six members (PRE1–PRE6) in Arabidopsis. The HLH motif of PRE members is a highly conserved motif that mediates protein-protein interactions among basic HLH proteins [46]. Unlike the true basic HLH proteins composed of at least 147 members in Arabidopsis [47], PREs lack the basic region near the motif, which is involved in DNA binding activity. Herein, the expression level of PRE1 and PRE5 was significantly higher in transgenic ZjGRF1 Arabidopsis plants than in WT (Fig. 3), possibly due to the low GA content in transgenic ZjGRF1 Arabidopsis, which acts as a compensation effect.

In Arabidopsis, SCL3 is a positive regulator of GA signal. The expression of SCL3 is induced by DELLA, but inhibited by GA. SCL3 interacts with DELLA to regulate root growth and seed germination by antagonizing the activity of DELLA protein [48, 49]. In this study, the expression level of SCL3 was significantly higher in transgenic ZjGRF1 Arabidopsis plants than in WT (Fig. 3), possibly due to the lower GA content in transgenic ZjGRF1 Arabidopsis (Fig. 1a).

Paclobutrazol is an inhibitor of GA synthesis and inhibits plant growth. In this study, the sensitivity of transgenic ZjGRF1 Arabidopsis to PAC decreased (Fig. 4).

However, the molecular mechanism by which GA regulates SOC1 expression is unclear. GA promotes the expression of LFY by binding to the cis-acting element on the LFY promoter [21, 50]. In contrast, SOC1 regulates the expression of LFY by directly binding to the promoter of LFY gene. Therefore, GA regulates the transcription of LFY through SOC1 dependent and independent pathways. GA may affect the flowering development of plants by regulating the expression of SOC1 and LFY in apical meristem. In this study, mRNA levels of LFY and SOC1 were lower in transgenic ZjGRF1 Arabidopsis than in WT (Fig. 5), possibly due to the lower GA content in transgenic ZjGRF1 Arabidopsis (Fig. 1a).

Conclusions
In this study, the role of ZjGRF1, 14-3-3 gene of Z. japonica, was identified in the GA signalling pathway. Therefore, these results can provide insights into further study of the function of 14-3-3 genes of seagrass.

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Authors’ Contributions SC designed the study and conducted the experiments. GQ conducted field sampling and identification. SC and GQ wrote the manuscript. All the authors reviewed the manuscript.

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Availability of data and material All data generated or analyzed in this study are included in this published article and supplementary file. The nucleotide and deduced amino acid sequence data of ZjGRF1 have been registered in the GenBank (No. MW199706).

Code Availability Not applicable.

Declarations

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

Ethics approval Not applicable.

Consent to participate Not applicable.

Consent for publication Not applicable.

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