Identification and characterization of genetic markers associated with salinity and abiotic stress tolerance in the Tetradium daniellii acclimatized in Uzbekistan

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Abstract. This study investigated the presence of genetic markers associated with salinity and stress tolerance in Tetradium daniellii using gel electrophoresis. Three markers (CAT, GR, RBOH) were identified out of 12 markers screened that are linked to salinity resistance and tolerance to drought, cold, pathogens and cadmium stress. The identification of these markers provides insights into the genetic basis of stress tolerance in T. daniellii.

1 Introduction

Tetradium daniellii, belonging to the Rutaceae family, is a medicinal plant distributed mainly in Southeast Asian countries. This species, which has a tree life form, is used as a source of medicine for the treatment of dermatitis, headache and stomach inflammation [1]. This species is acclimatized to many countries of the world (Korea, USA, Poland, Tajikistan, Uzbekistan, Vietnam, etc.) (POWO 2024). Abiotic stresses such as drought, temperature extremes, salinity, heavy metals, and UV radiation can reduce plant growth and productivity by more than 50% [2,3]. Currently, the pollution of the biosphere is accelerating due to the presence of heavy metals, which leads to soil and water pollution. When plants are planted in metal-contaminated soil, it causes significant environmental stress for the plants [4]. Heavy metal pollution, known for its high toxicity and long biodegradation, has become a major problem, especially in urban soils [5]. Heavy metals such as cadmium (Cd), which have a large atomic mass, have various harmful effects on the environment and ecosystem. Cd is highly toxic to many forms of life, it can enter the food chain and threaten human health. In the list of 20 most toxic elements, Cd ranks seventh and is classified as a potential human carcinogen [6]. In plants, even small amounts of Cd in the root medium, measured in micromoles, can cause toxicity [7]. Cd toxicity in plants inhibits root growth and photosynthesis, disrupts antioxidant defenses in all plant organs, and limits overall growth [8]. Cd also affects the water balance in plants [7]. The sensitivity

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of plants to these metals and their responses vary depending on the physiological and genetic composition of plants [8]. Under Cd stress conditions, NADPH oxidase plays a role in generating reactive oxygen species (ROS), which act as signaling molecules to activate plant defense mechanisms [9]. In addition, NADPH oxidase indirectly removes toxic Cd ions by activating proton pumps and creating a proton gradient across the plasma membrane [10]. NADPH oxidase (NOX) is an important enzyme expressed by the RBOH gene [11]. RBOH genes in plants have been extensively studied in various plant species [12]. A bioinformatic analysis of 22 plant species in 2023 by Zhang et al identified 181 RBOH homologues [13].

A lot of scientific studies have been conducted on genes that respond to abiotic stresses and *T. daniellii*, but no molecular studies have been conducted to study the resistance of acclimatized individuals to adverse abiotic stresses in the territory of Uzbekistan.

Our research is aimed at studying the existence of a group of genes related to response to abiotic stresses, namely 279 RBOH, 251 CAT, 263 GR genes in *T. daniellii* plant. This study aimed to identify genetic markers in *T. daniellii* associated with salinity and stress resistance that could provide insights into its stress tolerance.

2 Materials and Methods

2.1 Plant material collection

Fresh leaves of *T. daniellii* were obtained from Namangan city in Uzbekistan, where they had adapted to the local climate. Careful consideration was taken during the leaf selection process to ensure that only undamaged and disease-free leaves were chosen.

2.2 DNA extraction

The total genomic DNA was extracted from 20 mg of young leaf tissue using the modified CTAB (Cetyl Trimethylammonium Bromide) method, as described by Roullier et al. (2009) [9]. The extraction process was as follows:

1. The leaf samples were ground into a fine powder using liquid nitrogen to disrupt the cell walls and membranes.
2. CTAB extraction buffer, containing 2% CTAB, 1.4 M NaCl, 20 mM EDTA, 100 mM Tris-HCl (pH 8.0), 1% polyvinylpyrrolidone (PVP), and 0.2% β-mercaptoethanol, was added to the powdered leaf samples.
3. The homogenate was incubated at 65°C for 1 hour and 30 minutes to facilitate cell lysis and the release of DNA.
4. An equal volume of phenol/chloroform/isoamyl alcohol (24:24:1) was added to the homogenate and gently mixed by inversion. This step helped to separate the DNA-containing aqueous phase from the organic phase.
5. The mixture was centrifuged at 12,000 rpm for 10 minutes, and the aqueous phase was transferred to a new tube.
6. The DNA was precipitated by adding 40 µl of cold 3 M sodium acetate (pH 5.2) and 400 µl of 96% isopropyl alcohol, followed by incubation at -20°C for 3 hours. During this time, the DNA slowly settled out of the solution.
7. The sample was then removed from the refrigerator and centrifuged at 14,000 rpm for 10 minutes, causing the DNA to form a white precipitate.
8. The supernatant was discarded, and the DNA precipitate was purified by washing with 70% ethanol.

9. After centrifugation at 14,000 rpm for 15 minutes, the ethanol was removed, and the DNA was air-dried.

10. Finally, the DNA was dissolved in 30 µl of nuclease-free water.

11. The quality and quantity of the extracted DNA were assessed using a NanoDrop spectrophotometer, as shown in Fig. 1.

This detailed protocol outlines the step-by-step process of extracting high-quality genomic DNA from young leaf tissue using the modified CTAB method, as referenced in the original text.

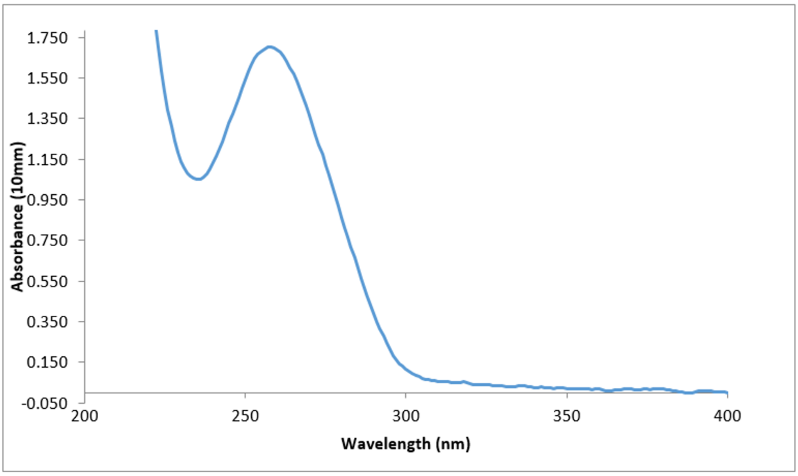

Fig. 1. The result of testing DNA quantity and quality using a nanodrop.

2.3 PCR Amplification and Gel electrophoresis

PCR was performed in a total volume of 20 µl containing 50 ng template DNA, 1X PCR buffer, 2.5 mM MgCl2, 0.2 mM dNTPs, 10 pmol each of forward and reverse primer, and 1 unit of Taq DNA polymerase. Thermal cycling conditions were: initial denaturation at 94°C for 2 min followed by 35 cycles of 94°C for 20 s, primer annealing at 55°C for 30 s, extension at 72°C for 30 s and a final extension at 72°C for 7 min. In our study, we used specific 12 different markers (Table 1) to target the tolerance genes. After completing the PCR process, the results were examined on a 1.5% agarose gel. To make a 100 ml gel, 1.5 g of agarose powder was weighed and placed in a flask. 1x TBE buffer was added until the volume reached 100 ml. The agarose was dissolved by heating at high temperature for 2-3 minutes, and then cooled to room temperature. Next, 4 µl of ethidium bromide was added to the gel, and it was poured into a special electrophoresis with a comb. Samples were loaded into the gel wells using 5 µl. The gel electrophoresis process lasted for 30 minutes. By running PCR products through an agarose gel and applying an electric current, we were able to observe the presence and size of the amplified DNA fragments

Table 1. Gene markers and their characteristics.

Markers name	Genes traits
253 CSP3	Cold resistance
254CYP707A	Drought and salinity tolerance
264 GST	Drought and salinity tolerance
278 PYL4	Drought and salinity tolerance

250 BCH	Drought and salinity tolerance
273 POD1	Drought and salinity tolerance
279 RBOH	Resistance to drought, cold, various pathogens and cadmium stress
251 CAT	Salinity tolerance
263 GR	Salinity tolerance
275 PP2C78	Salinity tolerance
276 PP2C8	Salinity tolerance
284 SOS2	Salinity tolerance

2.4 Sequencing and BLAST

The nucleotide sequences of the GR, CAT, and RBOH marker regions were obtained through Sanger sequencing of PCR amplicons. Sanger sequencing [10] is a chain termination method developed by Frederick Sanger in 1977 that remains the gold standard for DNA sequencing. In this approach, PCR is used to amplify the target region which is then sequenced using chain-terminating dideoxynucleotides that are labeled with different fluorescent dyes. The fragments are separated by capillary electrophoresis and detected with a laser, allowing the sequence to be reconstructed from the electropherogram trace file. For the BLAST search, the query sequences of 902 bp and 246 bp were input into the NCBI BLAST server [11] and compared against the Nucleotide Collection (Nr/Nt) database using the blastn algorithm optimized for nucleotide queries. This algorithm evaluates significance based on scores and E-values, with lower E-values indicating more significant matches. For both the 902 bp and 246 bp sequences, no matches were found below the E-value threshold of 1e-5, suggesting these sequences do not share significant similarity to any sequences currently deposited in the NCBI database.

3 Results and Discussion

Three genetic markers (CAT, GR and RBOH) were identified in the DNA of T. daniellii through gel electrophoresis (Fig. 2). The CAT marker presented as a clear band at 500 bp. CAT encodes the catalase enzyme, which directly decomposes hydrogen peroxide into water and oxygen [12]. Catalase plays a key role in protecting plants from oxidative damage caused by reactive oxygen species (ROS) accumulation under salt stress. The GR marker appeared as another clear band at below 500 bp. GR encodes the glutathione reductase enzyme that regenerates reduced glutathione (GSH) from its oxidized form (GSSG). GSH acts as an antioxidant to scavenge ROS. Maintaining adequate GSH levels via GR helps mitigate oxidative stress in plants exposed to salinity. A third distinct band was visible at 500 bp, representing the RBOH marker [13]. RBOH genes encode NADPH oxidase proteins involved in ROS generation during stress signalling [14]. ROS function as second messengers to trigger downstream antioxidant defences and stress responsive pathways in plants. The presence of RBOH confers resistance to various abiotic stresses through ROS mediated signaling.

The identification of CAT, GR and RBOH markers provides evidence of genetic traits for salinity tolerance and resistance to multiple stresses in T. daniellii. CAT and GR are associated with protection against salt-induced oxidative stress [12, 13]. RBOH contributes to ROS signaling during stress responses [14].

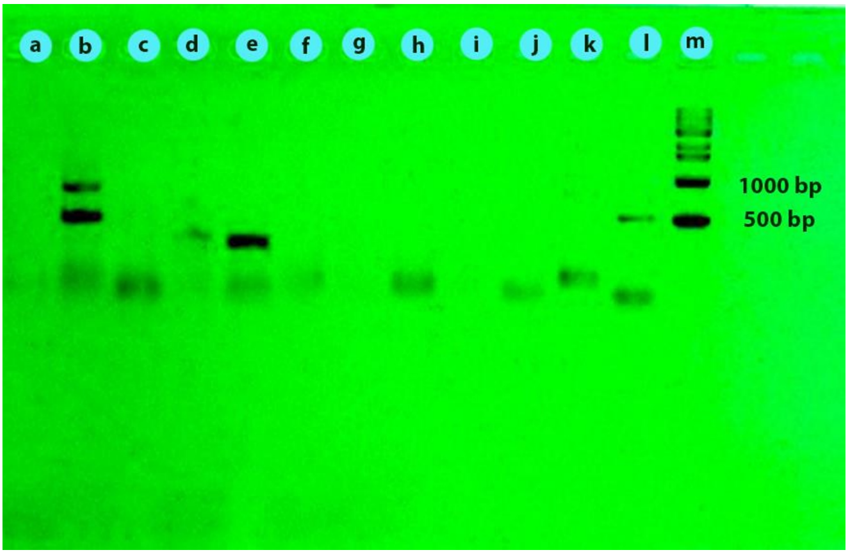

Fig. 2. Gel electrophoresis analysis of PCR results based on 12 different gene markers in *T. daniellii*: **a**. BCH; **b**. CAT; **c**. CSP3; **d**.CSP707A; **e**. GR; **f**. GST; **g**. POD; **h**.PP2C78; **i**.PP2C8; **j**. PYL4; **k**. SOS2; **l**. RBOH; **m**. DNA marker

The analysis of nucleotide sequences of GR marker regions in T. danielli has yielded significant insights when compared to the nucleotide sequences of plants possessing these genes (Fig. 3). The alignment of these sequences revealed a multitude of similarities, differences, and conserved regions within the CAT, RBOH, and GR marker regions. Notably, the GR marker sequence that we analyzed was found to have a high degree of similarity with a gene in Gossypium hirsutum, Theobroma cacao, and Mangifera indica plants. Specifically, the GR marker shared approximately 80% sequence identity with the corresponding genes in these other plant species. This high level of similarity suggests that the GR marker plays an analogous and conserved role across diverse plant lineages.

To further investigate the novelty of these sequences, we employed a BLAST (Basic Local Alignment Search Tool) search, a widely used bioinformatics tool that compares a query sequence to a vast database of known sequences to identify similar or homologous sequences. The BLAST search algorithm works by comparing the query sequence to sequences in the database and looking for short matches or "words" that are shared between the sequences. These word matches are then extended in both directions to maximize the alignment score based on matches and mismatches. The results of this BLAST search were intriguing, as no identical or highly similar nucleotide sequences were found in the database for the 902 bp and 246 bp sequences identified by the CAT and RBOH markers, respectively. This suggests that these sequences may be unique or have limited similarities to known sequences in the database, implying that they may be new or previously uncharacterized genes.

The absence of a matching sequence in the database with a high alignment score and low E-value may indicate that the sequences identified by the CAT and RBOH markers are novel or have not been previously described in other plant species. This finding has significant implications for the field of plant genetics, as it may lead to the discovery of new genes or genetic variants that play a crucial role in the biology of T. danielli and other related plant species. Further studies are needed to fully elucidate the functional significance and protein products of these sequences. Determining the specific roles of the putative CAT and RBOH genes could enhance our understanding of stress response pathways in T. danielli. Additionally, characterizing these novel sequences at the molecular level may uncover new targets for genetic engineering to develop stress-tolerant crop varieties through biotechnology approaches.

These findings offer insights into the plant's genetic basis of stress tolerance and its ability to thrive in saline coastal habitats. The presence of these markers indicates T. daniellii possesses genetic traits for withstanding salinity through antioxidant defence mechanisms mediated by enzymes such as catalase and superoxide dismutase. The RBOH marker also suggests the plant has pathways for ROS signaling to tolerate drought, cold, pathogens and heavy metal toxicity like cadmium. These adaptive mechanisms related to oxidative stress response and regulation likely underlie the plant's ability to thrive in saline coastal habitats under multi-stress conditions. Further research on the function and regulation of genes linked to these markers could help elucidate the specific physiological and molecular mechanisms that enable T. danielli to withstand the many abiotic and biotic stresses present in its natural habitat.

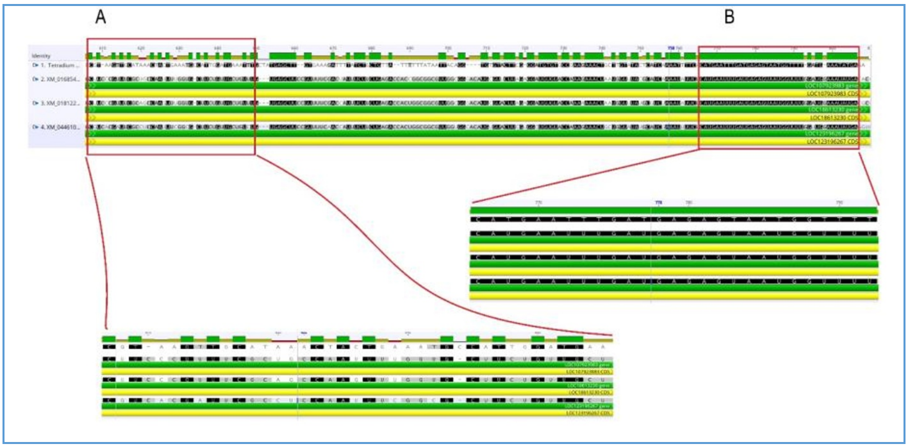

Fig. 2. The nucleotides detected by the GR marker in Tetradium danellii were compared with the nucleotide sequence of plants with these genes (Gossypium hirsutum, Theobroma cacao, Mangifera indica). Regions of high (A) and low (B) nucleotide diversity

4 Conclusion

This study identified three genetic markers (CAT, GR, RBOH) associated with salinity tolerance and resistance to multiple abiotic stresses in T. daniellii. The presence of these markers provides insights into the plant's genetic basis of stress tolerance. Specifically, the CAT and GR markers indicate T. daniellii possesses antioxidant defense mechanisms to mitigate salt-induced oxidative stress. The RBOH marker suggests it has pathways for ROS signaling to endure drought, cold, pathogens and heavy metals like cadmium.

Sequence analysis of the markers revealed high similarity between the GR marker in T. daniellii and corresponding genes in other plant species. However, the CAT and RBOH marker sequences did not match any sequences in databases, indicating they may be novel or previously uncharacterized genes. This significant finding warrants further investigation to elucidate the functional roles and protein products of these putative genes. Characterizing novel stress response genes could advance our understanding of T. daniellii's stress tolerance mechanisms.

The identified markers provide a genetic basis for T. daniellii's adaptability to saline coastal habitats under multi-stress conditions. The antioxidant defenses linked to CAT and GR likely enable survival under high salinity. Meanwhile, ROS-mediated signaling via

RBOH may underpin tolerance to various other environmental stresses. Further research on marker-linked gene expression and regulation could shed light on the specific physiological and molecular adaptations that allow T. daniellii to thrive in its natural habitat.

Overall, this study demonstrates that T. daniellii acclimatized in Uzbekistan possesses stress-linked genetic markers. The identification of these markers, including potentially novel candidate genes, provides valuable insights into the genetic determinants of the plant's stress tolerance traits. This work lays the foundation for future investigations aiming to elucidate the stress response pathways in T. daniellii at the molecular level. A greater understanding of these mechanisms could facilitate breeding efforts to develop stress-resilient crop varieties through biotechnology.

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