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Identification of a STOP1-like protein in *Eucalyptus* that regulates transcription of Al tolerance genes

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ABSTRACT

Tolerance to soil acidity is an important trait for eucalyptus clones that are introduced to commercial forestry plantations in pacific Asian countries, where acidic soil is dominant in many locations. A conserved transcription factor regulating aluminum (Al) and proton (H^+) tolerance in land-plant species, STOP1 (SENSITIVE TO PROTON RHIZOTOXICITY 1)-like protein, was isolated by polymerase chain reaction-based cloning, and then suppressed by RNA interference in hairy roots produced by *Agrobacterium rhizogenes*-mediated transformation. *Eucalyptus STOP1-like protein* complemented proton tolerance in an *Arabidopsis thaliana stop1*-mutant, and localized to the nucleus in a transient assay of a green fluorescent protein fusion protein expressed in tobacco leaves by *Agrobacterium tumefaciens*-mediated transformation. Genes encoding a citrate transporting *MULTIDRUGS AND TOXIC COMPOUND EXTRUSION* protein and an orthologue of *ALUMINUM SENSITIVE 3* were suppressed in transgenic hairy roots in which the *STOP1* orthologue was knocked down. In summary, we identified a series of genes for Al-tolerance in eucalyptus, including a gene for STOP1-like protein and the Al-tolerance genes it regulates. These genes may be useful for molecular breeding and genomic selection of elite clones to introduce into acid soil regions.

1. Introduction

The genus *Eucalyptus* contains many species, mostly native to Australia, that have a wide range of variation in different traits, including stress resistance (e.g., drought resistance) and industrial characteristics (e.g., cellulose fiber length) [1]. Because many species are fast growing and stress resistant, they are used in commercial afforestation for producing wood chips for pulp production [2]. In fact, *Eucalyptus* species have been introduced to plantations in Pacific, South Asian, South American and African countries, with a total of 17.9 million ha [3]. Although the genus is widely used, improvement of various stress resistance traits would be valuable for economic viability and the eco-friendly operation of commercial plantations [4,5].

For example, elite clones of eucalyptus for pulping plantations, which are usually fast growing and have suitable characteristics (e.g. longer cellulose fiber length) for pulp production, are sometimes more sensitive to environmental stress than naturally adapted accessions (e.g. [6]). Among the various stress factors, resistance to acidic soils is one of the most important traits for elite clones that are introduced to commercial plantations in Pacific Asian and South American countries because acidic soils are widely distributed in these regions [7]. Improved tolerance to acidic soil would have multiple benefits such as improving productivity, reducing the need for soil amendments such as limestone and phosphorus, and so on [8], when introducing varieties to these regions. Soil acidity often induces multiple stresses for crops, such as enhanced rhizotoxicity of Al^{3+} and H^+ [9], and deficiencies of alkaline bases (Ca and Mg, for example) and phosphate [8]. In

Abbreviations: ALMT1, ALuminum activated Malate Transporter 1; ALS3, ALuminum Sensitive 3; ART1, Al Resistance Transcription factor 1; GFP, Green Fluorescent Protein; GUS, β-Glucuronidase; MATE, Multidrug And Toxic compound Extrusion; STAR2, Sensitive To Al Rhizotoxicity 2; STOP1, Sensitive TO Proton rhizotoxicity1.

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general, native tree species are more tolerant than many crop plants to the complex stresses caused by soil acidity [10]. This tolerance is usually established by a long history of natural selection in particular sites with soil acidity. However, fast growing clones of *Eucalyptus* often show poor performance in biomass production and enhanced susceptibility to irregular because they were usually selected under different environments. Thus, resistance to soil acidity is one of the important target traits for fast growing clones of *Eucalyptus*.

The identification of genes regulated by the STOP1-like protein would be one approach to discover Al tolerance genes in *Eucalyptus* that can be used for molecular breeding and genomic selection. STOP1 (Sensitive TO Proton rhizotoxicity 1 [11]), a type of Cys2-His2 zinc finger protein, was identified in Arabidopsis (*Arabidopsis thaliana*) and regulates multiple genes for Al^{3+} and H^+ tolerance [11,12]. Subsequently, the rice ortholog ART1 (*Oryza sativa* Aluminum Resistance Transcription factor 1; RAP-DB: Os12g0170400), which regulates multiple Al tolerance genes in rice, was found [13]. These functional STOP1-like proteins were identified by mutant analysis in each plant species. Recently, reverse genetics using RNA-interference revealed that tobacco (*Nicotiana tabacum*) and moss (*Physcomitrella patens*) carry functional STOP1-like proteins that regulate Al-tolerance [14]. This suggested that a similar approach employing RNA-interference (RNAi) could be useful for identifying Al tolerance genes in eucalyptus.

In the present study, we isolated a gene for a STOP1-like protein from the hybrid-originated commercial clone GUT5 (namely EguSTOP1), and characterized it by *in planta* complementation assay and reverse-genetics using RNAi transformation. Through these approaches we found that the functional STOP1-like protein regulates a couple of major Al-tolerance genes of *Eucalyptus*, a MATE-citrate transporter [15] and an ALS3 [16] orthologue. This information could be useful to establish breeding strategies for *Eucalyptus* clones to be introduced to acidic regions.

2. Materials and methods

2.1. Plant materials and bacterial strains

The commercially planted eucalyptus clone GUT5, which was originally derived from a cross between *Eucalyptus grandis* and *E. urophylla*, was used in this study. This clone was maintained and propagated in vitro by cutting as described previously [17]. *A. thaliana* accession Colombia-0 (Col-0) was used as the reference wild type, and *ALS3*-KO (a T-DNA insertion mutant in *ALuminum Sensitive 3*; Salk: SALK_061074) was used as the host for *in planta* complementation assay. The hyper-virulent *Agrobacterium rhizogenes* strain ATCC15834 (American type culture collection, VA, USA) carrying a Ri-plasmid was used for obtaining transgenic hairy roots from GUT5. *Agrobacterium tumefaciens* strain GV3101 was used for transformation in *A. thaliana*.

2.2. Growth conditions

Cutting clones of GUT5 (2 cm shoots with 4–5 leaves, and 2 cm roots) were grown in vitro on floating plastic mesh (1.3 cm square with 50 holes per inch) in plastic pots containing 150 ml (per pot) of pre-culture medium consisting of 1/5 strength modified Hoagland–Arnon nutrients [0.4 mM $Ca(NO_3)_2$, 0.1 mM $NH_4H_2PO_4$, 0.2 mM $MgSO_4$, 40 μM KCl, 5.4 μM EDTA-Fe, 1 μM $MnCl_2$, 4.6 μM H_3BO_3, 0.076 μM $ZnSO_4$, 0.032 μM $CuSO_4$, 0.001 μM $(NH_4)_6Mo_7O_{24}$] and 1% sucrose at an initial pH of 5.6. The pots were kept at 23 °C with a 16 h photoperiod (20 μE m^{-2} s^{-1}). These plants were used for isolating RNA and analyzing citrate excretion. Transgenic hairy roots of GUT5 were put on agar media [1/4 strength B5 agar medium (1%, w/v agar) without P_i, containing sucrose (1%, w/v) (control, pH 4.6), and 100 μM $AlCl_3$ (Al toxic, pH 4.6; Al activities in the solution and at the plasma membrane (PM) surface were both 23.3 μM) or 25 μM $AlCl_3$ (Al toxic, pH 4.6; Al activities in the solution and at the plasma membrane (PM) surface were 5.8 μM and 7.7 μM, respectively, as estimated by the GEOCHEM-EZ [18] and SGCS (speciation-based Gouy-Chapma–Stern electrostatic) model program; http://www.uq.edu.au/agriculture/sgcs) or low pH (pH 4.0) medium] in plastic plates kept at a vertical angle for 3 days at 22 °C with a 16 h photoperiod (20 μE m^{-2} s^{-1}). In addition, *A. thaliana* was grown hydroponically in control (0 Al, pH 5.0) and Al toxic (2 μM Al added to the control solution, pH 5.0; Al activities in the solution and at the PM surface were estimated to be 0.3 μM and 10.2 μM, respectively, by the GEOCHEM-EZ and SGCS program) solutions as described previously [12]. The control solution consisted of 1/50 strength MGRL nutrients with 200 μM $CaCl_2$ and without P_i. Seedlings were grown under 12 h light (35 μE m^{-2} s^{-1})/dark cycles at 25 °C for 7 d, with medium renewal every 2 d.

2.3. Cloning of orthologous genes for ALS3, MATE and STOP1 from GUT5, and sequence analysis

Partial cDNA fragments of putative orthologous genes for ALS3, MATE and STOP1 were isolated by degenerate PCR with primer pairs used in previous studies (primer sequences are available in Sawaki et al. [19] and Ohyama et al. [14]). Full-length cDNAs were isolated by 3′ and 5′ RACE (rapid amplification of cDNA ends) as described by Kihara et al. [20]. Briefly, total RNA isolated from root samples was reverse transcribed into cDNA using a Transcriptor High Fidelity cDNA synthesis kit (Roche Applied Science, Tokyo, Japan) with oligo dT primers, which was then used for degenerate PCR and RACE. The ABI PRISM 3130xl DNA sequencer and ABI BigDye terminator system (ver 3.1) were used for DNA sequencing analysis according to the manufacturer's recommended protocols. Deduced amino acid sequence alignment and phylogenetic tree analysis were carried out in the GENETYX software version 11.01 (Genetyx, Tokyo, Japan) with ClustalW (http://www.ddbj.nig.ac.jp/index-e.html), and prediction of membrane spanning domains was carried out using HMMTOP (http://www.enzim.hu/hmmtop/). The sequences of the putative orthologous genes were deposited in GenBank under the following accession numbers AB826006 (*EguSTOP1*), AB826007 (*EguMATE1*) and AB826008 (*EguALS3*).

2.4. Vector construction and transformation

The GATEWAY® binary vector pGWB80 [21] was used to construct a mini-Ti plasmid for *EguSTOP1*-RNAi (RNA interference) (pGWB80-*EguSTOP1*-RNAi). Briefly, *EguSTOP1* fragments of about 250 bp were introduced into the two GATEWAY® cloning-sites designed in the T-DNA region that flanks the first intron of the *A. thaliana* isocitrate dehydrogenase gene (At1g65930). The GATEWAY® cloned sequences were oriented in the sense and antisense directions in the T-DNA. The pGWB80-*EguSTOP1*-RNAi was transformed into *A. rhizogenes* ATCC15834 by electroporation, and the strain was infected into in vitro grown GUT5 leaves to produce transgenic hairy roots according to the method described previously by Diets et al. [22] with some modifications [19]. Infected GUT5 leaves (1 cm square) were kept on 1/4 strength B5 agar (1%, w/v) medium containing galactose (1%, w/v) and acetosyringone (<20 μg/ml) for two days, and then washed in 6.25 mg/l of meropenem (Sumitomo Pharmaceutical Co., Tokyo, Japan) solution. The washed leaves were further incubated on 1/4 strength B5 agar medium (1%, w/v agar) containing sucrose (1%, w/v), meropenem