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Technical Note

A selection system for transgenic *Arabidopsis thaliana* using potassium thiocyanate as the selective agent and *AtHOL1* as the selective marker

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Abstract Concern about the use of bacterial antibiotic resistance genes as selectable markers is considered to be one of the factors in public resistance towards genetically modified crops. We had previously shown that the *Arabidopsis* AtHOL1 protein had high S-adenosyl-L-methionine-dependent methyltransferase activity toward the thiocyanate ion (NCS^-), which is toxic to plants beyond certain concentrations. Contrary to our expectations regarding the usability of *AtHOL1* as a selectable marker, our initial trial screenings for *AtHOL1*-overexpressing *Arabidopsis* on 1/2 MS agar medium containing various concentrations of potassium thiocyanate under normal growth conditions had been unsuccessful. In order to explore the possibility of using *AtHOL1* in *Arabidopsis* transformation, the screening conditions for *AtHOL1*-overexpressing *Arabidopsis* were further examined. We found that the transgenic seedlings could be screened when the seeds were germinated and grown under conditions of 7-day darkness followed by a 12-h light/12-h dark cycle for 3–7 days on a medium containing 3.0–5.0 mM potassium thiocyanate. Analyses of transgene insertions into the genomes and *AtHOL1* mRNA accumulation in the screened seedlings were also performed. Neither escaped seedlings nor altered growth was observed with the seedlings screened under the conditions reported here.

Key words: *Arabidopsis thaliana*, *AtHOL1*, potassium thiocyanate, S-adenosyl-L-methionine-dependent methyltransferase activity, selectable marker.

Selectable marker genes of bacterial origin, such as the neomycin phosphotransferase gene (*NPTII*) (Bevan et al. 1983) that develops kanamycin resistance in transgenic plants, have been widely exploited for plant engineering. However, since most of the antibiotic-resistant genes currently used in commercially available transgenic plants are of bacterial origin, there have been concerns about transfer of horizontal gene from transgenic plants back to bacteria. Strategies that enable transgenic plants to possess no marker genes (Ebinuma et al. 1997; Endo et al. 2002; Corneille et al. 2001; Cotsaftis et al. 2002; Dale and Ow 1991; Gleave et al. 1999; Goldsbrough et al. 1993; Komari et al. 1996; McKnight et al. 1987), marker genes of plant origin (Andersson et al. 2003; Ayalew and Stewart Jr 2005; Koizumi 2003; Okuzaki et al. 2007), or marker genes with no antibiotic resistance (Daniel et al. 2001; Erikson et al. 2004; Goddijin et al. 1993; Gough et al. 2001; Herrera-Estrella et al. 1983; Joersbo et al. 2003; Kunze et al. 2001; Perl et al. 1993; Rosellini et al. 2007; Weeks et al. 2000) could remove such reservations about transgenic plants.

In our previous biochemical studies, we have demonstrated the S-adenosyl-L-methionine (SAM)-dependent methyltransferase activity of *Arabidopsis* AtHOL1 protein (Nagatoshi and Nakamura 2007), which is a homolog of methyl chloride transferase (MCT) from *Batis maritima* (Wuosmaa and Hager 1990; Ni and Hager 1998) and thiol methyltransferase (TMT) from *Brassica oleracea* (Attieh et al. 2000; Attieh et al. 2002). The hypotheses concerning the physiological functions of these enzymes include their involvement in salt tolerance (Ni and Hager 1999) and in metabolizing glucosinolate hydrolysis products such as a bisulfide (HS^-) and a thiocyanate ion (NCS^-) (Attieh et al. 2000). In the course of our studies on *AtHOL1*, we found that 3-week-old *Arabidopsis* seedlings overexpressing *AtHOL1* had increased tolerance to potassium thiocyanate (KSCN), which is relatively inexpensive. Based on these findings, we examined a screening method for transgenic *Arabidopsis*, employing *AtHOL1* as a selective marker and KSCN as a low-cost selective agent. However, contrary to our expectation, screening of the transgenic

Abbreviations: CaMV, cauliflower mosaic virus; MS, Murashige and Skoog; NOS, nopaline synthase; SAM, S-adenosyl-L-methionine

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Arabidopsis plants of the T2 generation was unsuccessful when the plants were grown from seeds on a culture medium at 23°C under white fluorescent lights (*ca*. 30 μmol m^{-2} s^{-1}) (12 h-light/12 h-dark cycles) in a growth chamber. A similar method using TMT, a homolog of *AtHOL1*, had been published (Saini et al. 2005). Although the actual screening procedures were not given in detail, Saini et al. (2005) observed the differences in root development of the transgenic seedlings of several plant species including *Arabidopsis*, but assessed shoot development only in tobacco seedlings. These results raised the need for an appropriate screening condition that allowed facile identification of transgenic seedlings.

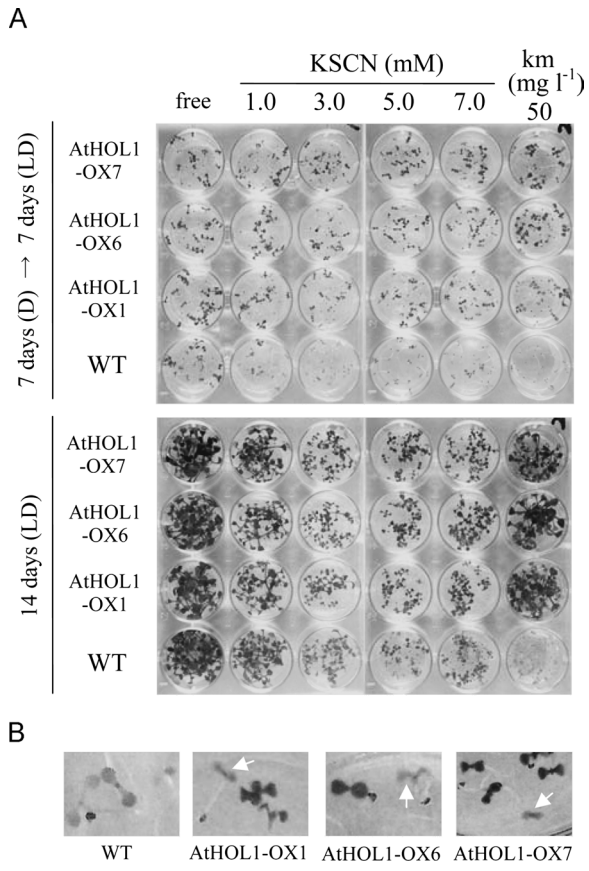

Figure 1. An examination of the screening conditions for *AtHOL1*-overexpressing *Arabidopsis*. (A) Wild-type seedlings and T2-generation seedlings derived from transgenic *Arabidopsis* lines (AtHOL1-OX1, AtHOL1-OX6, and AtHOL1-OX7) were screened on 1/2 MS medium (0.8% agar and 2% sucrose) containing KSCN (0, 1.0, 3.0, 5.0, and 7.0 mM) or Km (50 mg l^{-1}) under two light conditions. All the seeds were surface-sterilized and imbibed in the dark at 4°C for 2 days before sowing onto the agar medium in 12-well plates. After the stratification period, half of the seed set was grown in darkness for 7 days (D) followed by a 12 h-light/12 h-dark (LD) cycle for another 7 days (Top photo). The other half of the seed set was grown under the LD cycle for 14 days after stratification (Bottom photo). All the seedlings were grown at 23°C under white fluorescent lights (ca. 30 μmol m^{-2} s^{-1}) in a growth chamber. (B) Magnified pictures of seedlings on the selection media in panel (A). The T2 seedlings grown under darkness for 7 days followed by a 7-day LD cycle on media containing 5.0 mM KSCN. Arrows indicate seedlings with pale leaves possibly having no transgenes.

To establish the appropriate screening condition, *AtHOL1*-overexpressing *Arabidopsis* plants (AtHOL1-OX) were prepared by infiltrating *Arabidopsis thaliana* Col-0 with cultures of *Agrobacterium tumefaciens* LBA4404 (Bechtold et al. 1993) harboring a binary plasmid, pBI121-AtHOL1, containing *NPTII* driven by the nopaline synthase (NOS) promoter and *AtHOL1* driven by the CaMV35S promoter. The most appropriate KSCN and sucrose concentrations and light conditions were examined using the obtained transgenic *Arabidopsis* seeds (AtHOL1-OX1, AtHOL1-OX6, and AtHOL1-OX7) of the T2 generation that were a mixture of seeds both homozygous and heterozygous for the *AtHOL1* transgene. Examination revealed that wild-type and transgenic seedlings could be clearly distinguished when the seeds were germinated and grown on 1/2 MS medium containing 3.0–5.0 mM KSCN and 2% sucrose for 7 days under darkness, followed by a 12 h-light/12 h-dark cycle for another 3–7 days (Figure 1A). Cotyledons of both wild-type and transgenic seedlings appeared yellow and closed at the end of the first dark period. But during the second light period, the transformants began to show green and expanded cotyledons, while wild-type seedlings retained pale leaves (Figure 1B). Analyses using the Genevestigator online meta-analysis tool (Zimmermann et al. 2004) indicated that *AtHOL1* expression was high under light conditions and also at the germination and young seedling stages. These results

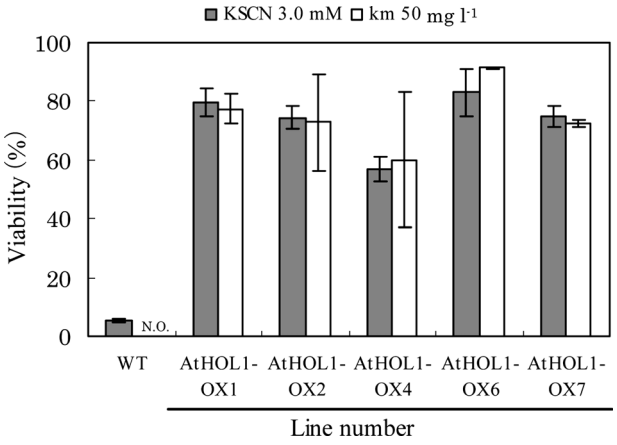

Figure 2. Comparison of the transgenic *Arabidopsis* screening efficiencies of the two marker genes. T2 seeds of five independent AtHOL1-OX lines were screened on agar media containing kanamycin (50 mg l^{-1}) or KSCN (3.0 mM). Seeds on the KSCN-containing medium were grown in darkness for 7 days followed by a 12-h light/12-h dark cycle for 7 days. The other growth conditions were the same as described in the legend of Figure 1. Seeds were grown on the kanamycin-containing medium under normal growth conditions for 14 days. The resistance of several thousand plants grown for 10-days was calculated on a per-plate basis by [plants with green cotyledons/germinated plants×100]. The averages±SE were from the results of three independent Petri dishes (9 cm in diameter) for KSCN- and kanamycin-resistance, respectively. The ratio of resistant to sensitive seedlings were not significantly different between wild-type and transgenic seedlings (P>0.05 by t-test). N.O., not observed.

implied that the dark growth condition suppresses endogenous *AtHOL1* expression in young seedlings permitting a lower endogenous *AtHOL1* activity and the resulting clearer identification of transgenic seedlings. Therefore, the first dark period could be important, at least in case of *Arabidopsis*, in order to screen transgenic seedlings germinated from seeds.

We then evaluated the screening efficiency of five *Arabidopsis* transformant lines of the T2 generation germinated on 1/2 MS medium containing either kanamycin or KSCN (Figure 2). The results indicated that the transgenic plants both homozygous and heterozygous for the *AtHOL1* transgene showed resistance to KSCN and a screening efficiency comparable to the kanamycin method. PCR amplification confirmed the insertion of *AtHOL1* transgenes into the genomes of ten seedlings from each of five independent transgenic lines (AtHOL1-OX1, AtHOL1-OX2, AtHOL1-OX4, AtHOL1-OX6, and AtHOL1-OX7) screened on the KSCN-containing medium. We quantified *AtHOL1* mRNA in five independent transgenic lines (AtHOL1-OX1, AtHOL1-OX2, AtHOL1-OX4, AtHOL1-OX6, and AtHOL1-OX7) using real-time PCR (Figure 3). *AtHOL1* mRNA accumulation in all of the examined transgenic lines was higher than that of wild type, though the mRNA accumulation varied considerably among the transgenic lines. These results suggested that the observed KSCN resistance is actually due to *AtHOL1* overexpression. Under the conditions used in this study, no apparent differences in growth and morphology were detected between wild-type and all the examined transgenic plants (Figure 4). These results indicate that *AtHOL1* overexpression together with the screening conditions examined here might be effective for a low-cost screening of transgenic *Arabidopsis* on KSCN-containing medium.

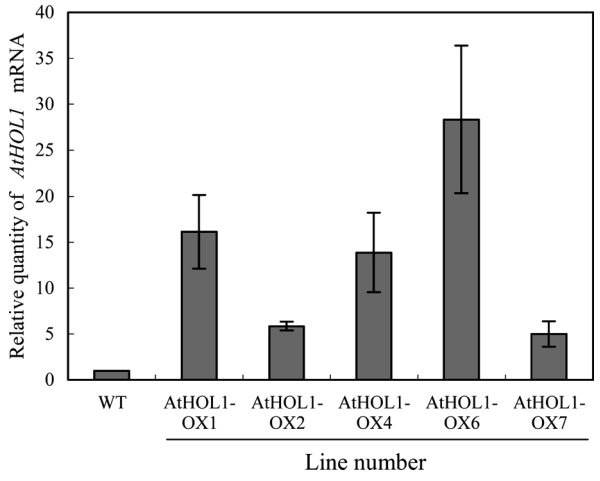

Figure 3. Quantitative analysis of *AtHOL1* mRNA by real-time PCR. T2 seeds of five independent AtHOL1-OX lines were screened on the KSCN (4.0 mM)-containing medium. The obtained resistant seedlings were grown on soil under normal growth conditions for 1 month. Total RNA was extracted from leaves of the *Arabidopsis* seedlings (*ca*. 0.2 g). cDNA was synthesized from total RNA using MMLV Reverse Transcriptase (Promega) according to the manufacturer's protocol. *AtHOL1* mRNA was quantified using the MX3000P real-time quantitative PCR system (Stratagene) and PCR reagent, SYBR Premix EX Taq (TAKARA). For each plant sample, the *AtHOL1* mRNA amounts were normalized with that of AtPP2AA3 (AT1G13320), an internal control (Czechowski 2005). The primer sequences used for *AtHOL1* amplification were 5′-CTTACGGCTCCTCACCAAAG-3′ (forward primer) and 5′-TGCTCTATGTATGTTTCAATGTTG-3′ (reverse primer), and those for *AtPP2AA3* were 5′-TAACGTGGCCAAAATGATGC-3′ (forward primer) and 5′-GTTCTCCACAACCGC TTGGT-3′ (reverse primer). *AtHOL1* mRNA amounts for each transgenic seedling were normalized with that in wild-type plants. The averages±SE were obtained from three independent plants.

Figure 4. Whole-plant growth phenotype of wild type and *AtHOL1*-OX7 plants. Appearance of wild type and a representative transgenic line (*AtHOL1*-OX7) were shown. Seeds of wild type and *AtHOL1*-OX7 were grown on 1/2 MS agar-medium for 10 days, and then the seedlings were grown on soil being fertilized with Hyponex under a 12-h light/12-h dark condition at 20°C for 40 days in a growth chamber.

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