Comparative analysis of various root active promoters by evaluation of GUS expression in transgenic Arabidopsis

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Abstract  To prepare various root active promoters for expressing transgenes and prevent gene silencing caused by the repeated use of the same promoter, the expression characteristics of various root active promoters were comparatively evaluated using GUS as a reporter gene. The high-affinity potassium transporter (HKT1;1), the Shaker family potassium ion channel (SKOR), the Shaker family inward rectifying potassium channel (AKT1), the major facilitator superfamily protein (MFS1), and the senescence associated gene 14 (SAG14) promoter from Arabidopsis (Arabidopsis thaliana) were used, and for comparison, four additional constitutive or green tissue specific promoters in the expression vectors were also employed. As the Gateway cloning technology provided by Invitrogen can offer high efficiency and cloning reliability, and easy manipulation of fusion constructs in vitro, our expression vectors are based on binary (destination) vectors compatible with this cloning technique. These destination vectors are also advantageous for stable expression of the transgene, as the heat shock protein terminator is utilized. The AtHKT1;1, SKOR, AKT1, MFS1 and SAG14 promoters were all active in roots but showed slightly different tissue specificities: AtHKT1;1, SKOR, and MFS1 were dominantly active in vascular bundle tissue, while AtHKT1;1 and MFS1—but not SKOR, AKT1, and SAG14—were active in root tips. SKOR showed the strongest root-specificity, and SAG14 showed the highest activity among the five root active promoters. The activity of MFS was developmentally regulated. These destination vectors are now available to express multiple transgenes in transgenic plants, especially in roots.

Key words: destination vector, expression vector, gene expression, GUS, root active promoter.

Transgenic technology is useful for the functional analysis of specific genes, and for molecular breeding techniques that impart specific traits. In such cases, the accurate characterization of promoters to express transgenes is important because, even if the same gene is used, the phenotype differs depending on its expression characteristics. For example, constitutive expression of the dehydration-responsive element binding protein 1 (DREB1) in transgenic plants caused both elevated drought, cold, and salt tolerance under the stressed conditions and growth retardation under non-stress conditions in transgenic plants; however, the stress-inducible expression resulted in the balancing of uninhibited growth under non-stress conditions and stress tolerance (Kasuga et al. 1999). Na⁺ transporters, including Arabidopsis AtHKT1;1, play important roles in plant salt tolerance (Berthomieu et al. 2003; Davenport et al. 2007; Sunarpi et al. 2005). Studies have shown that the constitutive overexpression of AtHKT1;1 was unsuccessful in producing salinity tolerant plants; however, overexpression of AtHKT1;1 specifically within the root xylem parenchyma cells of Arabidopsis led to improved Na⁺ exclusion and salinity tolerance (Møller et al. 2009). Similarly, the specific expression of AtHKT1;1 in root cortical and epidermal cells resulted in enhanced salinity tolerance in transgenic Arabidopsis and rice plants (Plett et al. 2010). Thus, the selection of an appropriate promoter is important for the foreign gene to perform the desired function.

Abiotic stresses are causing a significant loss of agricultural productivity worldwide, and soil salinity is one of the major concerns, particularly in irrigated soils (Horie and Schroeder 2004). Plant growth under salt stress conditions requires a tight control of K⁺ and Na⁺ uptake, long-distance transport, and accumulation, in addition to osmotic adaptation and removal of reactive oxygen species. Therefore, the improvement of plant salt tolerance through genetic engineering techniques requires the introduction of multiple transgenes with appropriate expression specificities (Wu et al. 2015). In
In this process, concerns arise in relation to the occurrence of transcriptional gene silencing (TGS), a phenomenon through which transgenes that share homology in their promoter regions are inactivated. As TGS can be substantially reduced by using different promoters to drive the expression of each transgene (Neuhuber et al. 1994; Thierry and Vaucheret 1996), it is important to prepare various promoters that show similar expression specificities. It has reported that increasing copy number of transgenes also cause gene silencing in plants (Davies et al. 1997; Ye and Signer 1996).

In this report, the purpose was to prepare various expression vectors with a promoter which is active in root tissues, and prevent gene silencing caused by the repeated use of the same promoter for expressing multiple genes of interest at once. In order to achieve this, five expression vectors, which are active in root tissue in Arabidopsis, were constructed and their expression profiles were comparatively characterized using β-glucuronidase (GUS) as a reporter gene. Additionally, one green tissue specific and two constitutive promoters were also constructed to compare the effects of transgenes when they were expressed constitutively or in shoot specific ways. To simplify the subcloning of the genes of interest into the various expression vectors using Gateway cloning technology (Invitrogen, CA, USA), a destination vector known as pGWB2 (Nakagawa et al. 2009) was used as original vector.

Root active promoters are indispensable to improve plant salt tolerance through the regulation of K⁺ and Na⁺ uptake and transport in roots. To express foreign genes in Arabidopsis roots, the promoters of five genes, which were reported to be expressed in Arabidopsis roots in different tissue-specific ways, were chosen. The expression (destination) vectors were constructed as follows: the nopaline synthase (NOS) terminator in the destination vector pGWB2 (Nakagawa et al. 2009) (Figure 1) was removed by digestion with restriction enzymes SacI and BamHI, followed by ligation with the Arabidopsis heat shock protein (HSP) terminator, which was excised from pR1201-AN (Takarabio, Ohtu, Japan) with restriction enzymes SacI and BamHI to construct pGH8 (Figure 1); then, pGH8 was digested with restriction enzymes Eco81I and XbaI, to remove the NOS promoter–neomycin phosphotransferase II (NPTII)-NOS terminator cassette and the cauliflower mosaic virus 35S (35S) promoter, followed by ligation with Eco81I-HindIII-XbaI linker to construct the promoter-less vector pGH8/EHX (Figure 1). Each promoter region of the following genes was amplified by PCR using the specific primers containing the recognition sequences of

![Diagram depicting the destination and GUS-expression vectors. Only the T-DNA regions are displayed; pGWB2 is the original destination vector. pGH8/EHX is the promoter-less intermediate vector used to construct the destination vectors. The numbers in parentheses indicate the length (kb) of each promoter sequence. 35S, AtHKT1;1, SKOR, AKT1, MFS1, SAG14, ACT2, ACT8, and Cab1 indicate the cauliflower mosaic virus 35S promoters and the promoters of the high-affinity K⁺ transporter, the Shaker family potassium ion channel, the Shaker family inward rectifying potassium channel, the major facilitator superfamily protein, the senescence associated gene 14, the actin2, the actin8, and the chlorophyll a/b binding protein 1 genes in Arabidopsis, respectively. Pro, promoter; NPTII, nopaline synthase; ter, terminator; attR1 and 2, site-specific attachment site 1 and 2, respectively; attB1 and 2, site-specific recombination site 1 and 2, respectively, produced by the LR recombination reaction; Cm’, a chloramphenicol resistance marker; ccdB, a cytotoxic protein; HPTII, hygromycin phosphotransferase II; HSP, Arabidopsis heat shock protein.](image-url)
HindIII or XbaI (Table 1) and the Arabidopsis (ecotype Columbia) genome DNA as template: the Shaker family potassium ion channel (SKOR, AT3G02850), which is localized in the stelar tissue of roots (Gaymard et al. 1998); the Shaker family inward rectifying potassium channel (AKT1, AT2G26650), which showed preferential expression in the peripheral cell layers of root mature regions and faint expression in the leaves (Lagarde et al. 1996); the major facilitator superfamily protein (MFS1, AT4G34950), which showed specific expression in root epidermis (Linn et al. 2017); and the senescence associated gene 14 (SAG14, AT5G20230), which showed specific expression in the inner stel of roots (Linn et al. 2017). Each amplified promoter fragment was digested with restriction enzymes HindIII and XbaI and was inserted into pGH8/EHX, which was digested with HindIII and XbaI to construct the destination vectors pSKOR, pAKT1, pMFS1, and pSAG14 (Figure 1). The destination vector pAtHKT1 containing the AtHKT1;1 promoter—which is specifically expressed in vascular bundle cells in the roots and shoots of Arabidopsis (Tada 2019)—and pGH1, containing the 35S promoter (Tada et al. 2019), were also used in this study. Destination vectors with a constitutive or shoot specific promoter were also constructed for comparison. The promoter regions of the constitutive expressing genes, actin2 (ACT2), actin8 (ACT8) (An et al. 1996), and of the green tissue associated gene 1 (AT1G49240), which showed specific expression in root epidermis (Linn et al. 2017); and the senescence associated gene 14 (AT5G20230), which showed specific expression in the inner stel of roots (Linn et al. 2017). Each amplified promoter fragment was digested with restriction enzymes HindIII and XbaI and was inserted into pGH8/EHX, which was digested with HindIII and XbaI to construct the destination vectors pSKOR, pAKT1, pMFS1, and pSAG14 (Figure 1). 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| Gene   | AGI code | Forward (5’→3’) | Revers (5’→3’) | Promoter length (bp) |
|--------|----------|-----------------|---------------|----------------------|
| SKOR   | AT3G02850| GCATTAAGCTTTGCCATATAAACCA | GTTATATGTCGATLGCGCGCGTGTG | 1083 |
| AKT1   | AT2G26650| AAAAGCTTCAAGCGTATACATGATAC | GTTCTCAGTTCTCTGTGACCTTC | 1833 |
| MFS1   | AT4G34950| ATAGCTTGCTCATCAGATCGCTGAAGCAC | ATCTAGATGTGTTGCGTGGACCACTCTG | 1800 |
| SAG14  | AT5G20230| TAGAAGCAGAGGCCTCTAGGACCC | GTCTAGAAGCACTATAGTCTGCGTCT | 818 |
| Actin2 | AT3G18780| CCAAGCTTTGACGTTCGGGACCCAGTGAC | ACTCTAGAGCATCGGCAATGGTCTATGAC | 1185 |
| Actin8 | AT1G49240| CCAAGCTTTGACGTTCGGGACCCAGTGAC | ACTCTAGAGCATCGGCAATGGTCTATGAC | 1125 |
| Cab1   | AT2G9930 | AGAAGTTGGTTGACTATCCTCCGTTCC | GTCTAGAGATTGCGACGCAAAAGTAGA | 1320 |

Underlined sections show the restriction enzyme (HindIII or XbaI) recognition sites.

Supplemented with 1% sucrose and 0 or 15 µg ml⁻¹ of hygromycin. The plants were grown at 23°C under a 16 h/8 h light/dark cycle with approximately 60 µmol m⁻² s⁻¹ light intensity.

Total RNA extraction from plant samples and real-time qRT-PCR analysis of the GUS gene were performed as previously described in Tada (2019); the GUS-specific primers GUS8R (5’-TCGTGCACCATCAGACGTTATCAG-3’) and GUS9F (5’-GGCCAAACGTGTTCTGATTAACCAC-3’) were used.

GUS enzyme activity in plants was detected histochemically using 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-Gluc) as substrate, and was quantified using 4-methylumbelliferoyl-β-D-glucopyranoside (4-MUG) as substrate as previously described in Tada (2019).

The promoter regions of the SKOR, AKT1, SAG14, MFS1, ACT2, ACT8, and Cab1 genes were amplified by PCR and the expression (destination) vectors pSKOR, pAKT1, pSAG14, pMFS1, pACT2, pACT8, and pCab1, were constructed, respectively (Figure 1). Along with the destination vectors pAtHKT1 and pGH1, which were previously constructed, each destination vector was subjected to reaction with the entry vector pENTR-GUS, via LR clonase enzyme, to be convert into GUS expression vectors. Arabidopsis plants (ecotype Columbia) were transformed with the expression vectors by floral dipping.

At least three independent T₂ transgenic lines with putative single transgenes, judged by segregation patterns of hygromycin-resistant and sensitive seedlings at T₂ generation, were subjected to a histochemical GUS assay using X-Gluc as substrate at both young (14-day-old) and reproductive (4-week-old) stages (Figures 2, 3). Strong GUS activity was detected in the vascular bundle tissue of roots, leaves, leaf petioles, and hypocotyls, and in root tips in the transgenic lines of the AtHKT1;1 promoter at the young stage (Figure 2A), but less activity was detected in these tissues at the reproductive stage (Figure 3A). These results were consistent with previous reports (Berthomieu et al. 2003; Mäser et al. 2002; Sunarpi et al. 2005; Tada 2019; Uozumi et al. 2000). In the SKOR lines,
Evaluation of root active promoters in Arabidopsis

GUS activity was detected strongly in the root vascular bundle and cortex tissues, and at a lesser extent in the vascular bundle in hypocotyls and leaf tips, but it was not detected in root tips at neither young or reproductive stages (Figures 2B, 3B). Those results were almost consistent with the report by Gaymard et al. (1998), in which SKOR expression was localized in the pericycle cells and in the xylem parenchyma cells. In the AKT1 lines, strong GUS activity was specifically detected in the entire root tissue except for root tips (Figures 2C, 3C), which is consistent with the report by Lagarde et al. (1996). In the MFS1 lines, GUS activity was detected in root vascular bundles, root tips, and leaves at the young stage, but the activity—especially root activity—was reduced to very low levels at the reproductive stage (Figures 2D, 3D). In the SAG14 lines, moderate GUS activity was detected in roots, hypocotyls and leaves at both young and reproductive stages (Figures 2E, 3C). On the contrary, MFS1 and SAG14 were specifically expressed in the root inner stele and epidermis, respectively, in previous report (Linn et al. 2017). The differences in promoter length and growth stage used in the former report and this study may explain the inconsistent tissue specificities. Thus, the AtHKT1;1, SKOR, AKT1, MFS1 and SAG14 promoters were all active in roots but their tissue specificities were different: AtHKT1;1, SKOR, and MFS1 were dominantly active in vascular bundle tissue, while AtHKT1;1 and MFS1—but not SKOR, AKT1, and SAG14—were active in root tips. Compared with the above mentioned promoters, the activities of 35S and ACT2 were detected in almost all tissues in roots and shoots at both young and reproductive stages (Figures 2F, 3C). On the contrary, 35S and ACT2 were detected in almost all tissues in roots and shoots at both young and reproductive stages (Figures 2F, 3C). 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reproductive stage.

Average GUS enzyme activities in shoots and roots in the AtHKT1, SKOR, AKT1, and MFS1 lines at the reproductive stage were highly correlated with their GUS transcriptional levels (Figures 4, 5). The order of intensity of GUS enzyme activities in the comparison promoter lines—35S, ACT2, and Cab1—was slightly inconsistent with the order of intensity of their transcription levels (Figures 4, 5). This inconsistence, however, was within the margin of error and was not significant. GUS enzyme activities in the shoots and roots of the AtHKT1, SKOR, AKT1, MFS1, and SAG14 lines were considerably lower than the activities in the comparison promoter lines, except for those in the roots of the SAG14 lines (Figure 5). GUS activities in the roots of the MFS1 lines were very low, matching the transcriptional levels at the reproductive stage (Figures 5, 4); SAG14 showed the highest activity in the roots, followed by SKOR, AKT1, AtHKT1;1, and MFS1 (Figures 4B, 5B). Though there have been reports of qualitative expression analysis of the AtHKT1;1, SKOR, AKT1, MFS1, and SAG14 promoters under different conditions, there have been no reports of any quantitative comparison of their expression activities. Their expression profiles and transcriptional activities were here analyzed in comparison with those of the 35S, ACT2/ACT8, and Cab1 promoters, under the same vector background (Figure 1). AtHKT1;1, SKOR, AKT1, MFS1 and SAG14 were active in roots but their activity levels were lower than those of 35S and ACT2 at the whole roots or shoots levels (Figures 4, 5). However, because 35S and ACT2 were active in most of the root cells—while AtHKT1;1, SKOR, AKT1, MFS1 and SAG14 were active only in specific cells—the activities of promoters in our destination vectors may be stronger than those of the constitutive promoters at the cellular level.

In summary, various expression vectors containing a root active promoter were developed. By using them in combination, it will be possible to express multiple transgenes in roots and prevent gene silencing caused by the repeated use of the same promoter. These promoters, however, showed slightly different tissue specificities in roots. Therefore, these destination vectors allow a more tissue-specific regulation of transgenes. As the expression vectors are based on binary (destination) vectors compatible with Gateway cloning, they are efficient, flexible, and simple to use. Furthermore, our destination vectors are advantageous for stable expression of the

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**Figure 4.** Transcriptional level of the GUS gene in transgenic plants. RNAs were extracted from shoots (A) and roots (B) in four-week-old plants (reproductive stage) grown on 1/2 MS agar medium and they were used for qRT-PCR. The expression levels of the ubiquitin extension protein were used for the normalization of GUS expression. Expression levels are shown relative to the expression of a AtHKT1;1 line in the roots. Each blue dot represents the mean of three biological replicates obtained from one transgenic line, and each orange dot represents the average of the values from three independent transgenic lines with one promoter-GUS construct. Inserted graph (A) shows enlarged view of the AtHKT1;1, SKOR, AKT1, and MFS1 lines.

**Figure 5.** GUS enzyme activity in transgenic plants. Crude protein extracted from shoots (A) and roots (B) in four-week-old plants (reproductive stage) grown on 1/2 MS agar medium, and used for the measurement of GUS enzyme activity. Fluorescence emitted by 4-MU, produced from 4-MUG by the GUS enzyme, was normalized per unit protein and per minute. Each blue dot represents the mean of three biological replicates obtained from one transgenic line, and each orange dot represents the average of the values from three independent transgenic lines of one promoter-GUS construct. Inserted graphs (A and B) show enlarged views of the AtHKT1;1, SKOR, AKT1, and MFS1 lines.
transgene, as they utilize the HSP terminator, which can support increased levels of foreign gene expression in plants when used in expression vectors (Nagaya et al. 2009). The constructed destination vectors and the expression profiling data are useful for expressing transgenes in transgenic plants, especially in roots, and analyzing functional of these genes.

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