tube RT-PCR and Pyrosequencing reactions to monitor the relative amounts of two alleles in a single sample helps to minimize variations due to RNA extraction and target amplification. There are several issues that may affect future applications of this technique to clinical samples. The study of a specific SNP may require additional characterization of the target by sequencing because of the inherent heterogeneity of RNA viruses. O'Meara et al. (8) have successfully utilized amplification oligonucleotides containing limited degeneracy at certain positions to accommodate annealing to a broad number of known HIV isolates because minor changes in the sequence can affect RT-PCR amplification of the target area, ultimately influencing the lower limits of detection for minor alleles. In addition, unexpected differences in the target can also affect results because the specific dispensation of nucleotides in the assay determines the identity of the SNP and the internal peak height controls (i.e., adjacent bases). Confirmation of the target sequence may be needed to ensure that both the SNP and the neighboring sequences are within the parameters of the analysis software. This approach should enhance both basic research on virus-host interactions and clinical analysis of viral dynamics in response to administration and/or cessation of therapies.

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Selection of Hygromycin-Resistant *Arabidopsis* Seedlings

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A large population of reporter gene-tagged *Arabidopsis* mutant lines is a powerful tool for use in functional genomics in the post-genome sequencing era. Introduction of transgenes has become easy, using the techniques of vacuum infiltration or floral dipping (1,2). Transgenes cloned into T-DNA vectors were introduced first into *Agrobacterium* and then into plants using these techniques. To select transgenic plants, antibiotic resistance, the gene for which is carried on the T-DNA, is commonly used for selection against sensitive seedlings. Hygromycin is one of the more popular antibiotics used for selection of transgenic *Arabidopsis*. However, the main problem with hygromycin selection is that it is toxic even to resistant plants during long exposure. Such exposure causes damage to the seedlings and abnormal development. To avoid these side effects, the period during which seedlings are grown on the selection plate should be as short as possible. Hygromycin causes retardation of sensitive seedlings and does not kill them. This makes it difficult to select resistant seedlings, and we usually have to wait 2–3 weeks for the true leaves of resistant seedlings to appear.

To make hygromycin screening more efficient, we tried several different selection media. Here we report a simple medium for the hygromycin selection of transgenic *Arabidopsis*. It gives a clear contrast between resistant and sensitive seedlings in one week. For the hygromycin selection, we used T1 seeds from *Arabidopsis thaliana* ecotype Col-0 plants that had been infected with *Agrobacterium tumefaciens* GV3101 (pMP90RK) containing a reporter gene tagging T-DNA vector pPCVICEn4HPT (3). The T1 seeds were surface-sterilized by incubating in 70% ethanol for 1 min, followed by treatment with 10% bleach with 0.1% Triton® X-100 for 10 min, and then rinsed with distilled water three times. After the sterilization, seeds were suspended in 0.1% agar solution and sowed onto selection medium. Essential types of selection medium were tested. We examined eight media. One was a germination medium (GM) containing 1× Murashige and Skoog mineral salts (Wako Pure Chemical Industries, Osaka, Japan), 0.8% Bacto agar, 1% sucrose, 1× vitamin mixture (Sigma, St. Louis, MO, USA), 0.5 g/L MES, pH 5.7, 20 mg/L hygromycin B (Roche Diagnostics, Basel, Switzerland), and 100 mg/L cefotaxime sodium salt (Wako Pure Chemical Industries) and modified GM by elimination of some components. These include GM without sucrose, mineral

salts and vitamins without pH adjustment, mineral salts and MES, mineral salts without pH adjustment, vitamins and MES, and vitamins without pH adjustment. The other was a basic agar medium that contained 1 mM KNO_3, 0.8% Bacto agar, 20 mg/L hygromycin B, and 100 mg/L cefotaxime sodium salt without pH adjustment. After sowing, plates were kept in the dark for two days at 4°C to induce germination and transferred to continuous white light at 22°C for five days.

As shown in Figure 1A, there were two types of seedlings on the basic agar medium plates. Larger seedlings (indicated by arrowheads) could be seen against a background of smaller ones. These larger seedlings seemed to be hygromycin resistant. We tested whether these big seedlings had the hygromycin resistance gene using genomic PCR. It was found that all the large seedlings did have the hygromycin resistance gene (data not shown). The transformation efficiency by using the basic agar medium plate after five days was 1.6%. However, seedlings grown on the GM plate and other modified GM plates showed almost no differences (Figure 1, B–H). To make clear the differences between resistant (arrowheads in Figure 1B) and sensitive (background) seedlings, they were grown for an additional five days until the true leaves emerged. After 10 days, only the GM plate could select resistant seedlings, and the transformation efficiency was the same as that of the basic agar medium plate at five days. In the case of using a GM plate without sucrose (Figure 1C), resistant seedlings were distinguishable from sensitive seedlings after 13 days; however, the transformation efficiency was 0.8%. By using other modified GM plates, resistant seedlings were indistinguishable from sensitive ones even after 13 days (Figure 1, D–H). The plates containing mineral salts allowed sensitive seedlings to grow (Figure 1, B–F), and the plates without mineral salts did not allow even resistant seedlings to grow (Figure 1, G and H).

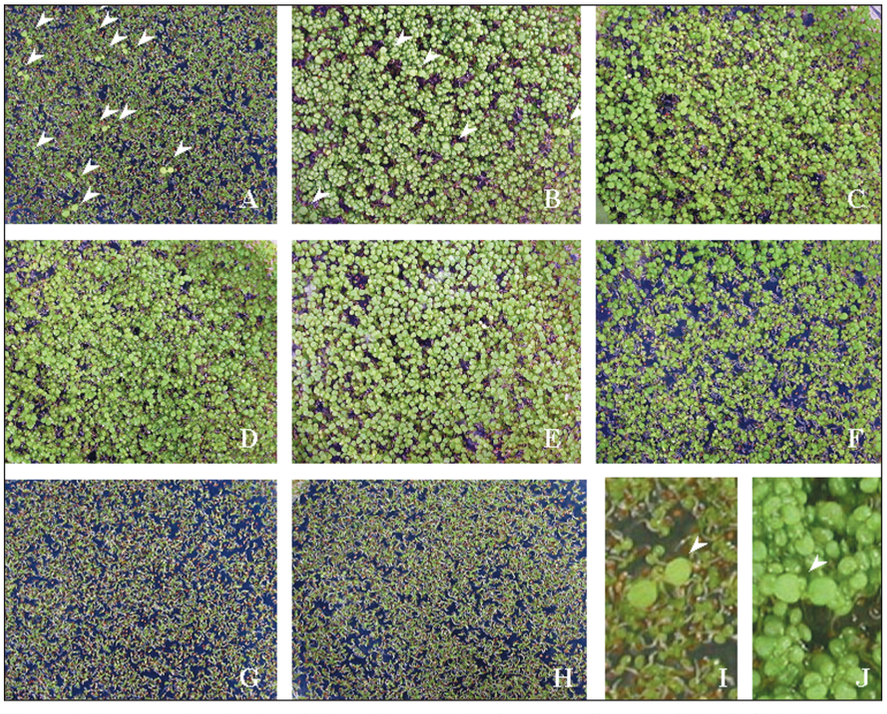

Figure 1. Hygromycin selection of T1 seedlings. (A) Basic agar medium. (B) GM. (C) GM without sucrose. (D) Mineral salts and vitamins without pH adjustment. (E) Mineral salts and MES. (F) Mineral salts without pH adjustment. (G) Vitamins and MES. (H) Media containing vitamins without pH adjustment. Each medium contained 20 mg/L hygromycin B and 100 mg/L cefotaxime. Panels I and J show hygromycin-resistant seedlings on basic agar medium and GM plates with higher magnification, respectively. Seedlings were grown under continuous white light at 22°C for five days. Arrowheads indicate hygromycin-resistant seedlings.

Although recent herbicide selection makes screening much easier, antibiotic selection in some cases still has some advantages; for example, plants weak at the seedling stage will be selected against by herbicide screening. By this method, we can clearly select resistant seedlings using antibiotic screening.

The method presented here will facilitate the screening and speed of construction of large-scale populations of *Arabidopsis* mutant lines for genome study and for conventional production of transgenic plants.

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