S36-006

Isolation of genes involved in stress tolerance by activation tagging

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Keywords: activation tagging, Arabidopsis, stress tolerance, paraquat, mutant

Introduction

Under both natural and agricultural conditions, plants are frequently exposed to stress. Plant productivity is strongly affected by environmental stress. Much of the injury to plants caused by stress exposure is associated with oxidative damage by active oxygen species (AOS) at the cellular level (Asada and Takahashi 1987, Foyer et al. 1994). The production of AOS is promoted by the exposure to a variety of environmental stress factors that include high light intensity, drought, extreme temperatures, high salinity, heavy metals, and a variety of toxins and herbicides. It has been reported that AOS-scavenging enzymes are induced by various stress conditions (Yoshimura et al. 2000). Accordingly, the use of gene transfer technology provides transgenic plants with altered levels of specific enzymes of the AOS-scavenging system with resistance under various stress conditions (Allen 1997, Miyagawa et al. 2000). On the other hand, plant cells may have many components involved in stress resistance except for AOS-scavenging enzymes; however, these are still unknown. We initiated activation T-DNA tagging to isolate mutants displaying resistance to a variety of kinds of environmental stress. This approach allows a discovery of novel genes in which recessive, loss-of-function mutations have no obvious phenotype. In this study, we report the isolation of a mutant exhibiting some stress resistance and the initial analysis of a sub-set of activation-tagged mutants.

Materials and methods

Materials---The binary vector, pPCVICEn4HPT (Walden et al. 1994), used to generate the activation T-DNA-tagging population and *Agrobacterium* GV3101 (pMP90RK), was obtained from Drs. R. Walden and T. Ichikawa (Max-Planck Institut für Zuchtungsforschung).

Plant material, growth conditions and transformation--- Arabidopsis thaliana (Columbia ecotype) was used for the experiments. The plants were grown in potting soil in growth chambers with a 16 h-day photoperiod (100 µmol m^{-2} s^{-1}), 20˚C night/23˚C day temperature cycle, and 60% relative humidity and watered with a standard nutrient solution. The binary vector pPCVICEn4HPT was introduced into *Arabidopsis* via *Agrobacterium tumefaciens* strain GV3101 (pMP90RK)-mediated *in planta* transformation according to the procedure of Chang et al. (1994).

Selection procedure--- Seeds from plants (T_0) were sown on MS plates (Murashige and Skoog 1962) containing 0.6 µM paraquat and grown for about 4 weeks under illumination at 100 µmol m^{-2} s^{-1} before transplanting the resistant plants to soil.

Plasmid rescue--- The plasmid sequences in pPCVICEn4HPT are flanked by several restriction enzyme sites that can be used for the rescue of T-DNA and the adjacent plant

sequences from transformed plants. The restriction enzymes *Eco* RI and *Kpn* I can be used for the rescue of sequences adjacent to the T-DNA. For plasmid rescue, 0.03 g of plant tissues was harvested, and the genomic DNA was prepared using the modified urea/phenol methods (Liu et al. 1995). The extracted DNA was resuspended in 300 µl of TE buffer (10 mM Tris-HCl and 1 mM EDTA, pH 8.0). After extracting the purified DNA twice with phenol-chloroform, 50 µl of the genomic DNA was digested overnight with the appropriate restriction enzyme in a 100-µl reaction mixture. After phenol-chloroform extraction, samples were ligated overnight at 14˚C in a total volume of 1 ml. The ligated DNA was precipitated; one-half was transformed by electroporation into *E.coli* DH10B, and one-tenth was used for the LA-PCR.

Results and discussion

We transformed *Arabidopsis* plants (Columbia ecotype) with a binary vector, pPCVICEn4HPT, to generate an activation T-DNA-tagging population. The *in planta* transformation method was used to generate approx. 20,000 seeds of transgenic plants. The T-DNA region of pPCVICEn4HPT contains four enhancers of the cauliflower mosic virus (CaMV)-35S promoter stacked near the right border and the selection marker hygromycin phsphotransferage gene (HPT) driven by the nopaline synthase promoter close to the left border (Walden et al. 1994). On the MS medium containing 0.6 µM paraquat under illumination at 100 µmol/m^2/s, wild-type plants could germinate but not grow. As shown in Figure 1, some mutants could grow and are shown to be green in color on the MS medium containing 0.6 µM paraquat, similarly to those under normal conditions. In the first screen of approx. 10,000 Columbia plants, we confirmed 32 dominant mutants with resistance to the paraquat treatment. These paraquat-resistant mutants (*pqr*) were analyzed by PCR using T-DNA internal primers to clarify the existence of the T-DNA in the genome DNA. As a result, we confirmed that all *pqr* mutants had T-DNA insertions.

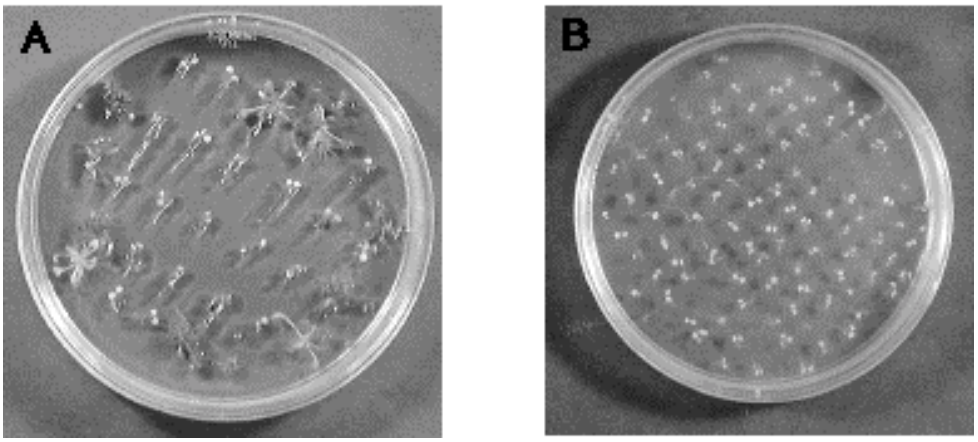

Fig. 1 Growth of activation-tagged mutants (A) and wild-type (B) on MS plates containing paraquat

To determine whether the mutant phenotypes were caused by overexpression of adjacent genes, we isolated and characterized the T-DNA insertions in several *pqr* lines. Plant sequences adjacent to the T-DNA insertions were recovered by the plasmid rescue or the LA-PCR with LB and RB specific primers. The extent of plant sequences isolated by the plasmid rescue or the LA-PCR ranged from a few hundred base pairs to around 6 kbp. Isolated DNA fragments were analyzed by DNA sequencing and comparison with the *Arabidopsis* genome DNA in the Kazusa *Arabidopsis* data-opening site (KAOS; http://www.kazusa.or.jp/kaos/).

The structures of five different insertion sites are characterized. In the *pqr-61*, *67*, *74*, *129*, and *130*, we identified the genes homogenous to thylakoid ascorbate peroxidase, cyt P-450, transport protein, kinesin-like protein, thyloid receptor interactor, Ser/Thr protein kinase, pactate lyase precursor, aldose 1-epimerase, and several unknown genes. To determine whether the mutant phenotypes were caused by overexpression of adjacent genes, we characterized the T-DNA insertions in several *pqr* lines. Now, we are determining the genes involved in paraquat resistance in the respective *pqr* mutants by micro array or Northern blot analysis. On the other hand, wild-type plants exhibited chlorosis under low-temperature and high-light conditions (2˚C, 1000 µmol/m^2/s). Among approx. 300 transformants, 3 dominant mutants did not show chlorosis under the same stress conditions (Fig. 2).

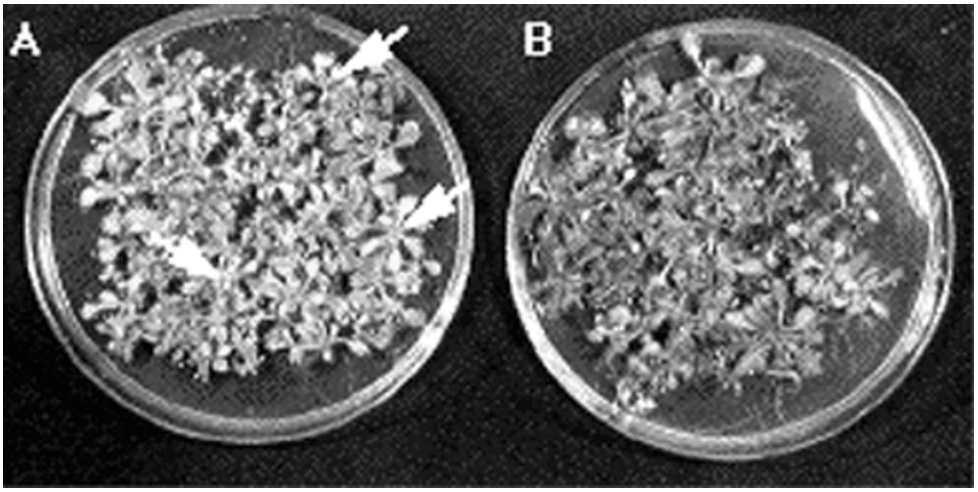

Fig. 2. Growth of activation-tagged mutants (A) and wild-type (B) on MS plates under low temperature (2˚C) and high light (1000 µmol/m^2/s) conditions. Arrows indicate the mutants that did not show chlorosis under these conditions.

Acknowledgments

We thanks to Drs. R. Walden and T. Ichikawa for providing plasmid pPCVICE4EnHPT. This work was supported by the New Energy and Industrial Technology Development Organization (NEDO) subsidized by the Ministry of Economy, Trade and Industry of Japan (METI).

References

Allen RD (1997) Free Radical Biology and Medicine **23**, 473-479.
Asada K, Takahashi M (1987) In Photoinhibition. Eds. Kyle DL, Osmond CB, Arntzen CJ. pp.227-287, Elsevier Scientific Publishers.
Chang SS, Park SK, Kim BC, Kang BJ, Kim DU, Nam HG (1994) Plant Journal **5**, 551-558.
Foyer CH, Lelandais M, Kunert KJ. (1994) Physiologia Plantarum **92**, 696-717.
Liu Y-G, Mitsukawa N, Oosumi T, Whittier RF (1995) Plant Journal **8**, 457-463.
Miyagawa Y, Tamoi M, Shigeoka S (2000) Plant and Cell Physiology **41**, 311-320.
Murashige T., Skoog F (1962) Physiologia Plantarum **15**, 473-497.
Walden R, Fritze K, Hayashi H, Miklashevichs E, Harlin H, Schell J (1994) Plant Molecular Biology **26**, 1521-1528.
Yoshimura K, Yabuta Y, Ishikawa T, Shigeoka S (2000) Plant Physiology **123**, 223-234