

# Phenotypic Screening of *Ds* transposon and *Activation-tag* Insertional Mutant Rice Population for Drought and Salinity Tolerant Related Traits

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## Abstract

The studies of rice genes through functional genomics are greatly facilitated by the availability of the complete genome sequences, including the complete physical map of the japonica rice cv. nipponbare. Using the *Ac* and *Ds* transposon, that are capable of transposition in various heterologous plants including the monocotyledon rice, combined with the enhancer element for generating activation tagged lines, it is possible to discover and isolate functional genes involved in various important agronomical traits; such as those involved in abiotic stress tolerance (drought, high salt) and biotic stresses diseases and pests. We have developed 1,785 first (T0) generation of mutant nipponbare rice by transposons *Ac/Ds* insertions containing *activation-tag*, generated by transformation using *Agrobacterium*-mediated method. Currently, we have generated approximately 1,000 stable lines with transposon *Ds* and *activation-tag* insertion ready for screening. Efficient screening methods for mutant Nipponbare rice lines have been established for agronomically important mutant traits. Among the new phenotypes related to important agronomical traits observed were drought and salt tolerant or sensitive, stunted and robust growth, variable root penetration and other interesting traits such as reduced tillering, rolled leaf and thin tiller.

**Keywords:** rice, insertion mutant, gene discovery, drought, salinity

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## Introduction

Drought is one of the major limitations to rice production. Improving drought tolerance and productivity are among the most difficult tasks for cereal breeders. The diverse strategies adopted by plants themselves to combat drought stress depend on the timing, severity and stage of crop growth. Drought stress induces a range of physiological and biochemical responses in plants, which include stomatal closure, repression of cell growth and photosynthesis, and activation of respiration (Shinozaki & Shinozaki, 2007). Plants also respond and adapt to water deficit at both the cellular and molecular levels, for instance by the accumulation of osmolytes and proteins specifically involved in stress tolerance. Drought stress may severely affect rice plant during generative stage, where flower

development is initiated. Therefore drought stress during generative stage will significantly reduced rice yield and may be one of the main cause of crop loss due to abiotic stress.

In addition to sensitivity to drought stress, rice is also a salt-sensitive crop (Grover & Pental, 2003). Excess salts may adversely affect all major metabolic activities in rice, which may cause cell wall damage, plasmolysis, cytoplasmic lysis, and damage to endoplasmic reticulum. Excess salts also reduce photosynthesis, and decreases germination and seedling growth (Khan *et al.*, 1997; Pareek *et al.*, 1997; Sivakumar *et al.*, 1998). These will lead ultimately to reduce growth and diminish grain yield, which will significantly contribute to grain yield losses. Relatively speaking, rice is considered as more sensitive to salts during early seedling than at

reproductive stages (Flowers & Yeo, 1981; Lutts *et al.*, 1995).

Rice insertional mutant populations harboring gene trap and activation tag have been generated (Nugroho *et al.*, 2006; 2007a), by transformation of Japonica rice cv. Nipponbarre using either the gene-trap (pNU400 and pUR224) or the activation tag construct (pMO22) (Nugroho *et al.*, 2006; 2007a). The mutant population was generated by using *Agrobacterium* transformation involving T-DNA carrying the two-component *Ac/Ds* transposon system, to enable fast development of large mutant population (Jeon *et al.*, 2000; Jung *et al.*, 2003; Hiei *et al.*, 1994). The activity of the *Ds* transposon to transpose in the presence of *Ac* transposon have been analyzed and confirmed (Nugroho *et al.*, 2007b). Using the available molecular markers (*GFP*, *hpt*, *bar* and *gusA*), stable *Ds* mutant have been screened and determined (unpublished data). These stable mutants can be used for phenotypic screening.

The advantages of the gene knockout/activation tagging approach for functional genomics include: 1) disruption of gene function leading to loss-of-function mutations, 2) activation of gene expression causing gain-of function mutations, 3) the technique being a direct way to determine the function of a gene product in situ, and 4) the inserted marker being available for subsequent identification of disrupted genes. The mutant population are phenotypically screened for traits related to drought and high salt responses and tolerant; such as tillering, weight, leaf quantity, leaf shape and root length. Semi large scale screening methods were used to screen for the drought and salt stress related phenotype. The first method developed was for drought responses screening using Polyethylenglycol 6000 (PEG600) which is a water limiting agent, to induced water stress. The second method used NaCl to create high saline environment. This paper describes our program in developing rice insertional mutant population and the identification of mutant phenotypes related to drought and salinity responses and tolerances.

## Materials And Methods

**Mutant population.** Population of mutants used in this experiments were obtained from

the rice insertion mutant collection of the Rice Research Group at the Research Center for Biotechnology, Indonesian Institute of Sciences (LIPI). Plasmid pUR224 and pNU400, and pMO22 were obtained from Dr. Narayana Upadhyaya (CSIRO, Plant Industry) and Dr. Andy Pereira (PRI, Wageningen University), respectively.

**Phenotyping by Drought screening.** Two methods were employed. The first method used PEG 6000 to screen for drought related phenotypes. Seeds were washed with sterilized water 3 times followed by application of 70% ethanol for 5 minutes. The seeds were then sterilized in 20% sodium hypochloride solution containing 3 drops of Tween 20 for 30 minutes. The seeds were then washed 3 times in sterilized water. Sterilized seeds were germinated in open bottles (15 seed/bottle) containing 25 ml Yoshida solution and placed inside closed transparent plastic boxes for 1 week. Aeration was provided by holes on each sides of the box. Drought treatment was done by growing the 1 week-old seedlings in a 25ml Yoshida solution (Yoshida *et al.*, 1976) containing 15% Polyethylenglycol 6000 (PEG6000) in the bottles. To maintain relative humidity, the bottles were kept inside aerated closed transparent plastic boxes containing NaCl saturated water. The Yoshida growth solution was replaced every week. Drought related phenotypes were scored 2 weeks after the drought treatment, including number of leaf, total weight, plant height and root length as well as the percentage of germination. The second method looked at root penetration. Seeds were surfaced sterilized as described above and germinated in a petri dish containing Yoshida solution. After 14 days the seedlings were transferred onto plastic cup containing sterilized soil with 3 mm thick wax bottom, placed on top of another plastic cup containing Yoshida solution. The ability of root to penetrate were observed after 14 days.

**Phenotyping by high-salinity screening.** High-salinity screening was conducted based on procedure described previously (Gregorio *et al.*, 1997) with minor modification. Seeds were germinated in Yoshida solution. After 7 days, seedling were transplanted into a hole in a styrofoam board supported by nylon net floating on a plastic box filled with Yoshida solution. The nutrient solution was replaced

every week. Salinization was initiated by adding 3 g NaCl/l nutrient solution 14 days after transplanting. After one week in the saline solution, the salt concentration was raised to 6 g NaCl/l and maintained for 14 days. Salt stress symptoms were evaluated. Salt injury at seedling stage was observed for symptoms such as number of leaf, total weight, plant height and root length.

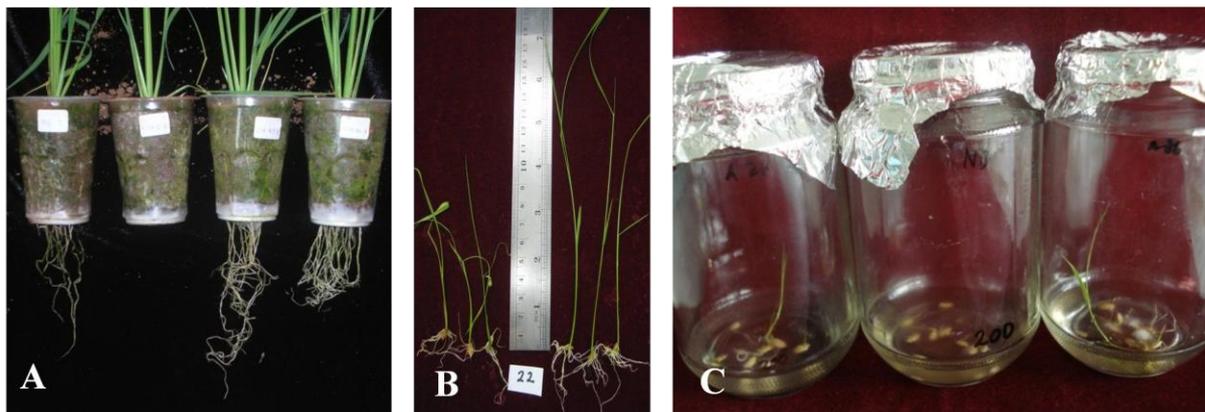
## Results and Discussion

### Drought tolerant mutant traits

Our observation indicated that there are mutants exhibiting phenotypes related to drought responses and tolerant. There are mutants with different root architecture and penetrability obtained. As shown in Figure 1A, among these mutations are longer or shorter root, variable branching root, thicker root and

low penetration root system. There are also mutants with higher ability to grow under 15% PEG6000, which may indicate ability to grow under drought environment (Figure 1B, C).

Plant-type traits such as tiller number and plant height modify the expression of secondary and integrative traits by affecting transpirational demand (Kamoshita *et al.*, 2008). Genotypes with greater plant height are often larger in overall plant size, and they tend to intercept more light and use water faster by transpiration, leading to lower plant water status (Kamoshita *et al.*, 2004), higher leaf death scores, and more spikelet sterility (Kato *et al.*, 2007). Mutants with related plant-type traits have also been observed such as robust growth (Figure 2A), dwarf (Figure 2B), low tillering (Figure 2C, D), and low seedling vigour (Figure 2E).



**Figure 1.** Drought and salinity tolerant related mutant phenotypes observed. A. Root shape/length, B. PEG tolerant, and C. PEG tolerant.



**Figure 2.** Drought and salinity tolerant related mutant phenotypes observed. A. Robust growth, B. Dwarf, C and D. Reduced tillering, and E. Low seedling vigour.

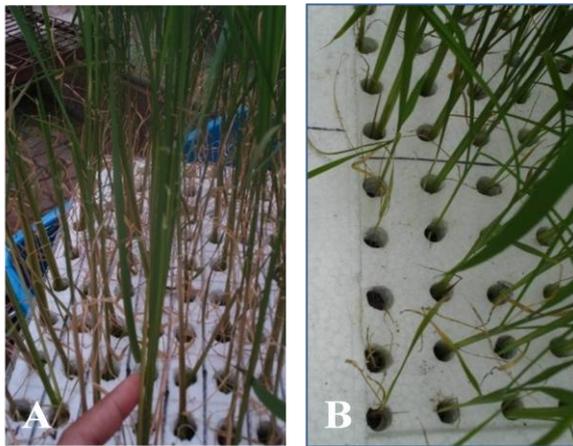
In principle drought tolerant traits can be divided into primary traits, secondary traits, integrative traits, phenology, and plant-type traits (Kamoshita *et al.*, 2008). Primary traits are further divided into constitutive traits (e.g., rooting depth, root thickness, branching angle, and root distribution pattern; Lafitte *et al.*, 2001; Kato *et al.*, 2006), and induced traits

(e.g., hardpan penetration and osmotic adjustment). Constitutive traits were expressed continuously even if there is no drought stress, while induced traits are expressed during drought stress. Constitutive root traits have a large effect on extractable soil water during drought, which will influence the expression both induced and secondary traits such as

maintenance of plant water status, canopy temperature, leaf rolling score, and leaf death score (e.g., Lilley & Fukai, 1994). These secondary traits may then reduce spikelet fertility and yield components (i.e., integrative traits), and ultimately, yield (Kobata *et al.*, 1994).

### Salt tolerant mutant traits

Excess salts adversely affect all major metabolic activities in rice including cell wall damage, plasmolysis, cytoplasmic lysis and damage to ER, reduction in photosynthesis, and overall decline in germination and seedling growth (Pareek *et al.*, 1997; Sivakumar *et al.*, 1998). The condition will ultimately lead to reduced growth and diminished grain yield. Rice is considered as more sensitive to salts stress during early seedling than at reproductive stages (Flowers & Yeo, 1981; Lutts *et al.*, 1995). Therefore, observation of the effect of salt on rice during seedling stage will give desirable results. Some mutants exhibiting salt stress responses obtained includes salt tolerant (Figure 3A) and sensitive (Figure 3B) mutants.



**Figure 3.** Drought and salinity tolerant related mutant phenotypes observed. A. Salinity tolerant and B. Salinity sensitive.

### Conclusion

Population of rice activation tag and gene-trap insertional mutant have been established. The population are still being expanded to obtain a more representative mutant numbers. This mutant resources offer excellent tools for both basic and applied researches, not only of rice but also of other cereals. This insertional mutant database facilitates identification and

isolation of genes that are functional in biochemical pathways, physiological processes, or growth and development of cereal crops. Many of these genes could be valuable for crop improvement; such as, to enhance biotic and abiotic stress tolerance, productivity, and quality of cereal crops, through genetic engineering approaches. So far, some potential mutants have been identified through our initial screenings, such as responsiveness to drought and salinity stresses. These mutants could provide invaluable information to the genetic bases of drought and salt stress tolerant. The success of this project would give a major impact on the development of agriculture biotechnology in Indonesia.

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