Simple yet stringent screening methodologies for evaluation of putative transformants for abiotic stress tolerance: salt and cadmium stress as a paradigm

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ABSTRACT

Rigorous and stringent screening methodologies to select transformants at both seedling and plant level under cadmium or NaCl stress were developed. At seedling level, two screening strategies were standardized. One involved germination on filter paper/agar in the presence of either CdCl₂ (125 µM) or NaCl (350–450 mM) for 9 days and selection of tolerant putative transformants. The other involved germination of the seedlings on soilrite by irrigation of 450 mM NaCl. Further, at plant level, *in vitro* evaluation for stress tolerance involved a simple leaf senescence bioassay. Combination of the seedling and plant level screening strategies would result in the initial identification of promising transformants for further analysis. [Physiol. Mol. Biol. Plants 2010; 16(2) : 115–121] E-mail : rohinisreevathsa@rediffmail.com

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INTRODUCTION

Transgenics for abiotic stress tolerance provides an option to develop cultivars that are tolerant to abiotic stresses. Lot of effort is being directed towards the development of transgenic crops for various abiotic stresses like drought, salinity, heavy metal tolerance etc. Screening strategies that can be followed to select high performing transgenics are important. In this direction, the methods used to impose stress, severity and duration of stress, the parameters to be measured and how the observed responses of the plant fit into an overall strategy for resisting the stress hold considerable importance. Evaluating the stress responses of transgenic plants is most challenging because it not only identifies the plants that thrive under stress but also gives an idea whether the plant's overall performance has been altered.

There are a number of screening methods for quantifying resistance of plants to several abiotic stresses (Verslues *et al.*, 2006). Screening for stress tolerance

either precedes or follows molecular analysis. Research groups follow different approaches in order to primarily identify the putative transformants with probable efficiency to combat stress. Selection of plants that are termed to be salt tolerant is dependent on many parameters like leaf proline and soluble sugar content (He *et al.*, 2007), sodium ion concentration (Ohta *et al.*, 2002; Bao *et al.*, 2009), root proton export capacity (Zhao *et al.*, 2006), relative water content (Brini *et al.*, 2007; Bao *et al.*, 2009), net photosynthetic rate (Zhao *et al.*, 2006; Bao *et al.*, 2009), leaf surface determination (Brini *et al.*, 2007). However, less cumbersome, easy to handle and high throughput initial screening strategies will always be advantageous. In our lab, we have found it an advantage to evaluate and assess putative transformants before taking them through PCR and other molecular analysis. Imposition of such a pre-screening test has distinct advantages as described by Zhao *et al.* (2006) for rice transformants harboring *NHX1*. In our experience (unpublished data), such a screening before molecular analysis always results in the identification of a higher percentage of transgenics than after molecular analysis. Therefore, it would not only be important to screen for stress tolerance initially but also

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be rigorous. In this paper, we describe stringent and rigorous screening strategies for selection of putative transformants with focus on two abiotic stresses, salinity and cadmium tolerance.

MATERIALS AND METHODS

Plant material

Two rice genotypes, Vikas and Azucena were used in this study. Rice cv. Vikas was transformed with two genes *NHX1* (Na^+/H^+ antiporter) and *AVP1* (*Arabidopsis* Vacuolar Pyrophosphatase) for salt tolerance following *in planta* transformation protocol (Kumar *et al.*, 2009) whereas, Azucena was transformed with *pcs* (*Phytochelatinsynthase*) for Cd tolerance. The seedlings with just emerging plumule were infected by pricking at the meristem with a sterile needle and immersed in a suspension of *Agrobacterium* for 1 h. Then, the seedlings were washed briefly with sterile water and transferred onto autoclaved soilrite (vermiculite equivalent) moistened with water for germination under aseptic conditions in the growth room in wide mouth capped glass jars of 300 ml capacity with 5 seedlings per jar. After 5 to 6 days, the seedlings were transferred to soilrite in pots and were allowed to grow under growth room condition for at least 10 days before they were transferred to the greenhouse. The growth room was maintained at 26–28 °C under a 14 h photoperiod with a florescent light intensity of $35 \mu \text{mol m}^{-2} \text{s}^{-1}$. The T_1 seeds of the above transformants were used for the development of screening strategies.

Screening strategies

T_1 seeds were surface sterilized with 0.1 % HgCl_2 for 10 min and 0.5 % Bavistin. Screening strategies were developed based on the kind of abiotic stress. Concentrations of the stress inducers were standardized earlier with the wild type. Three levels of screening have been elucidated.

- 1. Screening at seedling level:** Two-day old seedlings of both putative transformants and wild type were used to screen on NaCl and CdCl_2 . Initially, as an optional step, for screening of transformants with the *AVP1* gene, the seedlings were induced with 250 mM NaCl for 3 h. Later, the induced and non-induced seedlings were incubated for nine days in the presence of 350 mM NaCl and $125 \mu \text{M CdCl}_2$. Earlier, the wildtype seedlings were subjected to 150, 250, 350, 450 mM NaCl and 75, 100, 125 and 150

$\mu \text{M CdCl}_2$ to arrive at the screening concentration. After nine days, the seedlings were recovered on distilled water for three days. The root and shoot length of the screened seedlings were measured and the seedlings that showed root and shoot lengths atleast 10 % more than the wild type were considered for further analysis.

- 2. Irrigation with the stress inducer:** Initially, to standardize the concentration of the stress inducer, 2 day old seedlings of the wild type were irrigated with different concentrations of NaCl (150, 250, 350, 450 and 500 mM). Based on the standardization, 450 mM NaCl was chosen. Later, putative transformants along with the wildtype were shifted to plastic bowls containing soilrite and maintained in the green house. They were irrigated with Hoagland's medium containing 450 mM NaCl at an interval of 3–4 days and scored for symptoms. Symptoms included chlorosis, tip burning and reduction in root and shoot lengths.
- 3. Screening at plant level by stress induced leaf damage:** Leaf discs from leaves of 4–6 weeks old putative transformants and appropriate control (wild type) were transferred to either liquid MS salts or placed on filter paper discs dampened with MS salts or placed on solidified MS salts on 0.6 % agar containing $125 \mu \text{M CdCl}_2$ and 450 mM NaCl. They were incubated for 72 h and the extent of bleaching and chlorophyll loss was determined. Chlorophyll estimation was carried out following the protocol of Arnon (1949).

RESULTS AND DISCUSSION

Along with the development of transgenics to combat abiotic stress, the screening for effective transgenics is equally important. Larger the number of transgenics, rigorous should be the screening. Generation of large number of transgenics is possible by *in planta* transformation strategies that avoid tissue culture. *In vitro* analysis of plants to check for stress tolerance plays a very important role that eventually leads to the selection of plants for further field assessment.

Yamaguchi and Blumwald (2005) have emphasized the need for routine stress tolerance screens to obtain potential salt-tolerant lines from among the putative T_1 transformants. This procedure has been generally followed in many studies including those dealing with overexpression of genes for ion homeostasis (Zhao *et*

al., 2006; Chen *et al.*, 2007). These techniques have been reviewed by Ohta *et al.* (2002) and Chen *et al.* (2007). Generally two approaches are being followed, viz., screening before molecular characterization and following it. In the first approach, there were disadvantages as observed by Chen *et al.* (2007) with *NHX1* in Rice. In this case, there was no difference between the wild type and transgenic plants in 100 mM NaCl. It was also observed that transgenic and control seedlings recovered and resumed normal growth after salt treatment of 200 mM NaCl. Therefore the total shoot length, after 15 days growth was taken as a criterion for salt tolerance and in this criterion the

transgenics were superior. A radically different approach was followed by Zhao *et al.* (2006) with transgenic rice carrying a vacuolar antiporter, *NHX1*. Molecular analysis for confirmation of gene integration was carried out after four generations of screening on salt stress. This would result in larger co-relation between the efficacy and molecular analysis. The second approach therefore would be the choice because of the stringency. In the present paper, we report high throughput and rigorous screening of rice transformed with three genes, *pcs* for cadmium tolerance and *NHX1*, *AVP1* for salt tolerance. Screening strategies were standardized at both seedling and plant level.

Fig. 1. Screening at the seedling level by germination. (a) Extent of seed germination and relative root and shoot growth (b) for wild type and transgenic plants harboring the *pcs* gene after nine days of germination on 125 μ M CdCl₂ and recovery for three days. (c) Extent of seed germination and relative root and shoot growth (d) for wild type and transgenic plants harboring the *NHX1* gene after nine days of germination on 350 mM NaCl and recovery for three days. (e) Extent of seed germination and relative root and shoot growth (f) for wild type and transgenic plants harboring the *AVP1* gene after induction on 250 mM NaCl for 3 h and continued on 350 mM NaCl for nine days of germination followed by recovery for three days. WTT: wild type treated; PT: putative transformants.

Seed germination assays was the first level of screening. It was standardized for both salt and cadmium tolerance. Two day old seedlings were placed on 0.6% agar containing either 125 μ M CdCl₂ which induces Cd stress or 350 mM NaCl which induces salt stress. Differential germination of the T₁ seeds was seen in both the treatments *i.e.*, CdCl₂ (Figure 1a) and NaCl (Figure 1c) by nine days of incubation followed by recovery for 3 days. The effect of the stress was highly evident from root and shoot lengths taken at the end of recovery period, both in the case of CdCl₂ (Figure 1b) and NaCl (Figure 1d). This indicated not only the stringency of selection but also the probability of transformants at the germination level. Further, in the screening of transgenics over expressing the *AVP1* gene, an additional but optional step was included wherein the seedlings approximately 36 h old with their radicle just emerging were subjected to an induction concentration of 250 mM NaCl for 3 h (Figure 1e and 1f). This step was thought to reduce the escapes which could be more often seen in such screening strategies. Therefore, induction of the just germinating seedlings with an intermediate concentration of the stress inducer before shifting to the standardized screening concentration for nine days followed by a recovery phase for three days can be followed as a primary step in the

screening for abiotic stress tolerance. Though seed germination assays can provide a quick idea of stress response, it must be interpreted with caution. A high rate of germination under salt stress might not be well correlated with salinity tolerance at later developmental stages (Almansouri *et al.*, 2001; Saleki *et al.*, 1993; Kurth *et al.*, 1986). As an alternative another screening strategy was used in transgenics raised with *NHX1* gene, which involved direct irrigation of salt to growing seedlings. Two day old germinating T₁ seedlings of rice cv. Vikas transformed with *NHX1* gene were shifted to soilrite and irrigated with 450 mM NaCl as described in materials and methods. With continued irrigation for 2 weeks, there was a significant difference seen in the continuation of the growth of the seedlings that could tolerate salt concentration of 450 mM when compared to the treated wild type and the non-transformants. The tolerant seedlings were green and fresh where as the susceptible plants showed chlorosis and ultimate death (Figure 2a). This observation corroborated in the root and shoot lengths of these seedlings (Figure 2b & 2c). This strategy therefore can be adopted as another primary screening strategy for abiotic stress tolerance. But one important consideration is that plasmolysis should be avoided (Munns, 2002). For this, pot grown plants should be added with salt gradually and allow

Fig. 2. Screening at the seedling level by irrigation. Difference in the growth of wild type and transgenic seedlings harboring *NHX1* (a) and relative root and shoot (b and c) when irrigated with 450 mM NaCl for 2 weeks. WTC: wild type control; WTT: wild type treated. L1-L3: putative transformants.

the plants to adjust. Pots should be periodically re-watered with the same saline solution to maintain salt concentration. Further, these experiments like the agar based seedling screening should have wildtype treated in similar way. Another important aspect of this screening strategy is the maintenance of nutrient content in the medium of growth and the addition of salt does not cause deficiency of other ions like calcium. In the present study salt was suspended in Hoagland's medium to maintain nutrient balance.

Therefore, the above two strategies are not only stringent but also rigorous in the selection of putative transformants. The plants selected in both the strategies were shifted to the green house. These plants were allowed to recover and establish for 3-4 weeks. They were later subjected to the next level of screening based on the target gene incorporated. This strategy utilized leaves of 4-6 week old plants. This leaf disc bioassay is an example of a quick method to measure salt/Cd-induced damage (Singla-Pareek *et al.*, 2003; Sanan-Mishra *et al.*, 2005; Singla-Pareek *et al.*, 2006). This technique is not only simple and rapid but also eliminates any effect of altered root to shoot ion transport, as it allows a more focused assessment of the ability of the tissue to tolerate Na^+ accumulation. Leaf

discs from the three independent transformants and the appropriate wild type were subjected to stress in three different media. In the case of rice transformants with *pcs* gene, they were floated on a solution of $125 \mu\text{M}$ CdCl_2 (Figure 3a) as well as placed on filter paper dampened with $125 \mu\text{M}$ CdCl_2 (Figure 3c). The leaves from the plants harboring the *NHX1* (Figure 3e) and *AVP1* (Figure 3g) genes were placed on 0.6 % agar media containing 450 mM NaCl suspended in quarter strength Hoagland's medium and full strength MS salt respectively. On observation after 72 h, there was a difference in the response of the transformants (Figure 3 a, c, e and g) and wild type to the stress imposed. Leaves of the treated wild type and the susceptible plants were totally bleached whereas the leaves from the tolerant plants retained the colour as in the wild type control. The chlorophyll estimation from these leaves corroborated with the response (Figure 3 b, d, f and h). Therefore, the plants with chlorophyll content on par with the wild type control was considered as tolerant and selected for further analysis.

The present study, therefore, brings together a few stringent high throughput screening strategies and presents an effective mode for the identification of putative transformants. All the strategies together could

Fig. 3. Screening at plant level by stress induced leaf damage. Retardation of stress-induced senescence (a & c) and the chlorophyll content (b & d) in leaves of transgenic rice plants overexpressing *pcs* by floatation and on filter paper disc with $125 \mu\text{M}$ CdCl_2 respectively. (e) & (g). Response of the leaves to salt stress and their chlorophyll content (f) & (h) in transgenic plants expressing *NHX1* and *AVP1* genes on quarter strength Hoagland's medium and full strength MS medium respectively containing 450 mM NaCl. WTC: wild type control; WTT: wild type treated. PT: putative transformants.

Fig. 3. continued

be used as an effective screening protocol, where the strategies one and two form the primary screening followed by the leaf bioassay which acts as the secondary screening. These screening protocols can then be followed by measurement of a number of traits that quantify stress response. These include measurement of growth and survival to assess the overall level of stress tolerance. However, this study emphasizes on the high throughput screening strategies that can be effectively used as a primary tool for selection of putative transformants.

Identification of genes by exploiting the genomic approaches has to go hand in hand with development of better, more rigorous methodologies to evaluate the large number of putative transformants in the T_1 stage. It is imperative to therefore impose rigorous and effective methodologies to drastically narrow down the potential tolerant lines. This would immensely benefit the molecular biologist/biotechnologist/physiologist involved in developing transgenics for abiotic stress tolerance. Consolidation of stringent primary screening strategies as a part would help in identification and better understanding of the phenotypes associated with abiotic stress tolerance.

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