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Development and Standardization of a Robust Protocol to Screen for Cellular Tolerance Contributing for Abiotic Stress Tolerance in Rice

SHIVALINGAPPA BANGI, M. S. PARVATHI AND K. N. NATARAJA
Department of Crop Physiology, College of Agriculture, UAS, GKVK, Bengaluru-560 065
e-mail : nataraja_karaba@yahoo.com

Abstract

Scarcity of water is a severe environmental constraint which is affecting the productivity of staple crops like rice. There are many strategies that can be adopted to enhance the stress tolerance potentials of crops, among which manipulation of cellular tolerance traits has been demonstrated to be advantageous in recent studies. There are methodologies available to screen the superior transgenic lines/germplasm accessions; however, information on reliable combinational approaches that aim in testing the plant responses to multiple stresses is limited. In the study, a robust multiple abiotic stress screening approach has been standardize to evaluate plant types based on their cellular tolerance capacity. A gradual multi-stress induction protocol was used to screen multigene expressing rice transgenic lines and growth data was subjected for Z-distribution analysis. To standardize and validate the protocol, rice transgenic plants co-expressing three different transcription factors as a model system we used. The protocol developed can be used to screen large population of transgenic plants for cellular tolerance as demonstrated in the study. The approach can also be used to select superior plant types with efficient cellular tolerance mechanisms.

Keywords: Cellular tolerance, Abiotic stress, Transgene, Z-distribution

INCREASING human population and associated climate change is a serious threat to meet food demand. Therefore, concerted efforts are being made towards improving specific traits associated with growth and productivity in diverse crop plants (Lesk *et al*., 2016). Amongst various abiotic stresses limiting crop productivity, drought, salinity and high temperature pose serious problems. In future, occurrence of drought events are likely to increase land degradation in arid and semi arid regions and crops could suffer up to a 50 per cent yield loss if a drought event occurs at the reproductive growth stage (Venuprasad *et al*., 2007). Crop biologists are now trying to pyramid traits to enhance cellular tolerance capacity with an aim to sustain productivity in various field crops (Tang *et al*., 2019; le-Roux *et al*., 2019; El-Esawi *et al*., 2019; Gonzalez *et al*., 2019 and Li *et al*., 2019). In tropical regions, rice is one of the important staple food crops. To save irrigation water, semi-irrigated aerobic rice cultivation is being encouraged in traditional rice growing areas. Breeders are trying to identify/evolve rice varieties with drought tolerance traits, as the incidence of drought episodes seems to be common in aerobic rice cultivation. Crop breeding programs involve screening and evaluation of a large germplasm for stress tolerance, which require rapid screening and evaluation protocols.

In recent years, many approaches have been proposed for targeted improvement of traits in field crops. There are reports on identification of elite donor germplasm accessions and pyramiding traits using molecular breeding and/or transgenic approaches in different crops (Pruthvi *et al*., 2017; Gonzalez *et al*., 2019 and Li *et al*., 2019), including rice (Parvathi *et al.*, 2015). Most of the transgenic approaches are generally adopted by using single gene linked to the specific cellular tolerance trait (Hong *et al.,* 2016 and Xu *et al.,* 2015). Transgenic lines have been developed using specific transcription regulators in various field crops such as rice (Karaba *et al.,* 2007), maize (Nelson *et al.,* 2007 and Nguyen *et al.,* 2013), wheat (Saad *et al.,* 2013), potato (Bouaziz *et al.,* 2013), groundnut (Pruthvi *et al*., 2017) and perennial crops like mulberry (Sajeevan *et al*., 2017). Since tolerance to many abiotic stresses such as drought is governed by multiple traits

(Huang *et al*., 2010 and Parvathi & Nataraja, 2016), single gene transgenics may not yield desirable results. From this context, in recent years, attempts were made to generate transgenic plants that co-express multiple genes contributing for cellular tolerance under stress (Parvathi *et al*., 2015 and Pruthvi *et al.,* 2017). The success of evaluating such transgenic lines lies in the efficient and accurate screening of the putative transformants, which can be indicative of the contributory significance of the multiple genes employed. The aptness of the approach used for screening transgenic lines depends upon identification and evaluation of superior events during early generations using different comparative cellular tolerance assays. There are methodologies developed for screening germplasm accessions/transgenic lines for cellular tolerance by Temperature Induction Response (TIR), which employs a gradual temperature increase approach to select superior lines (Senthil Kumar *et al*., 2003). However, there are no combined approaches developed to screen plant types for improved cellular tolerance. From this context, the major goal of the study was to develop and standardize a robust multi-stress-induction technique to identify the plant types with superior cellular tolerance in response to stress. Rice transgenic plants co-expressing three different transcription factors have been used as a model system to optimize the protocol. The method demonstrated could be used to identify the superior transgenic lines or germplasm accessions to screen for superior cellular tolerance capacity types.

Material and Methods

Plant material

The rice (*Oryza sativa* L., cultivar, AC39020) transgenic seed materials co-expressing the stress responsive genes *AhBTF3*, *AhNF-YA7* and *EcSAP-ZF*, along with *PsAKR*1, the glyphosate resistant selection marker (Parvathi *et al*., 2015) were used in the present study. The seeds were germinated on petriplates and two-day old uniform healthy seedlings were used for the experiments.

Development of screening approach to select lines with superior cellular tolerance

The approach was developed by integrating data collected from different abiotic stress screening treatments. The data collected was subjected to Z-distribution analysis for identification of superior transgenic lines.

Abiotic stress screening

Two-day old transgenic rice seedlings were exposed to three different stresses - Sodium Chloride (NaCl) induced salinity, Poly Ethylene Glycol (PEG) 8000 induced drought stress and Cadmium Chloride ($CdCl_2$) induced heavy metal stress, by gradual induction method at three different concentrations. Induction stress would activate the cellular processes contributing for cellular tolerance. For gradual induction of NaCl induced salt stress, the seedlings were subjected to 100 mM NaCl for 24 h, followed by 200 mM NaCl for 24 h and 300 mM NaCl for 48 h. Similarly, PEG-8000 induced desiccation stress was imposed gradually using -4 bar (24 h), -6 bar (24 h) and -8 bar (72 h) osmotic potentials consecutively. The inductive conditions for $CdCl_2$ induced heavy metal stress were 400 µM (24 h), 600 µM (24 h) and 800 µM (48 h) consecutively. After the stress period, shoot and root length (cm) was recorded, mean value and standard deviation was calculated and used for Z-distribution analyses.

Data analysis for identification of superior transgenic lines

Seedling growth data collected was subjected for Z-distribution analysis using the formula, Z-value ={(General mean - Specific mean) / Standard deviation}. The values calculated using data from any two stresses were plotted as X-Y scatter to distribute the data points in four quadrants (Ramu *et al.,* 2012).

Characterization of selected transgenic lines

a) *Glyphosate tolerance assay* : The transgenic plants used in the study have ability to detoxify, herbicide glyphosate. To identify transgenic nature of the selected superior lines, seedlings were raised in

small plastic cups of 2:1 garden soil and sand mixture for 15 days under green house conditions. The seedlings were then swabbed with glyphosate (750 ppm) and monitored for glyphosate-induced injuries / symptoms.

b) *Confirmation of gene integration* : Genomic DNA was extracted from 25-day old transgenic rice seedlings using Cetyl Trimethyl Ammonium Bromide (CTAB) method (Doyle & Doyle, 1987). Polymerase chain reactions were carried out in a 20 µl reaction using 1.0 µl template DNA, 1.0 µl each of gene specific forward and reverse primers of marker gene, *PsAKR*1*,* 5 µl master mix (pre-mix containing Taq DNA polymerase, dNTPs, and $MgCl_2$) and 12 µl nuclease free water in DNA thermal cycler (Master cycler Gradient, Eppendorf AG, Germany) separated on agarose gel and visualized using gel documentation system (Herolab, GmbH Laborgerate, Germany).

c) *Transgene expression* : Total RNA was isolated from selected superior transgenic lines (Sajeevan *et al*., 2014) and used for first strand cDNA preparation using the Moloney-Murine Leukemia Virus reverse transcriptase (MMLV-RT; MBI Fermentas, Hanover, MD, USA). The cDNA was used as template to perform semi-quantitative RT-PCR to study the marker gene expression under standardized conditions (Nethra *et al*., 2006 and Parvathi *et al.,* 2015), using marker gene (*PsAKR*1) specific primers.

d) *Evaluation of cellular tolerance capacity of superior transgenic rice lines* : Two days old seedlings of the selected superior transgenic lines were subjected to mannitol (200 mM) for one week in agar media to induced osmotic stress for evaluating the cellular tolerance capacity of the superior transgenic lines and the per cent change in growth over the wild type was calculated.

Results and Discussion

Cellular tolerance mechansims are considered as important traits associated with stress tolerance in plants (Raju *et al*., 2014 and Parvathi & Nataraja, 2016). Concerted efforts are being made to improve cellular tolerance using diverse approaches to sustain crop productivity under stressful conditions. Many genes linked to cellular tolerance traits have been prospected and used for targeted crop improvement using transgenic approaches (Yu *et al*., 2013 and Pruthvi *et al*., 2014). Although considerable improvement has been made in plant transformation methods, the screening of transgenic plants is often a laborious work and in many situations, researchers fail to identify the true transgenic events, if the traits under consideration are difficult to phenotype. Evaluation of cellular tolerance traits requires a good knowledge on the inter-linked physiological processes and appropriate screening methodologies. From this context, an attempt has been made in the study to develop a screening protocol to identify superior plant type/s exhibiting desirable cellular tolerance under different abiotic stresses. Multi-stress induction protocol was developed to identify seedlings with improve cellular tolerance. The transgenic plants selected for the study co-express three different transcription factors, which are known to improve cellular tolerance (Parvathi *et al*., 2015). Two-day old rice seedlings of wild type and 23 different transgenic lines were subjected to NaCl, PEG 8000 and $CdCl_2$ induced stresses by gradual stress induction protocol, to disturb cellular processes and also to create an opportunity for acclimation response. Root growth of different plant types in response to the different induction stresses are represented in Fig.1. Since gain-in-function transgenic lines were used to test the hypothesis, the lines used for the study showed superior phenotype compared to wild type. However, there were differences in growth responses amongst the 23 transgenic lines used. Growth data collected using different lines are presented in Fig. 1.

To identify the lines with superior tolerance to the stresses imposed, growth data was subjected for Z-distribution analysis. Generally, the genotypes with higher Z-values for parameters under consideration will have highest negative Z-value and will fall in the 3rd quadrant (-, -). The plants which show least values for both parameters will have highest positive Z-value, and will fall in 1st quadrant (+, +). The plants which show more negative trend in one parameter and positive in another parameter will fall in the 2nd quadrant

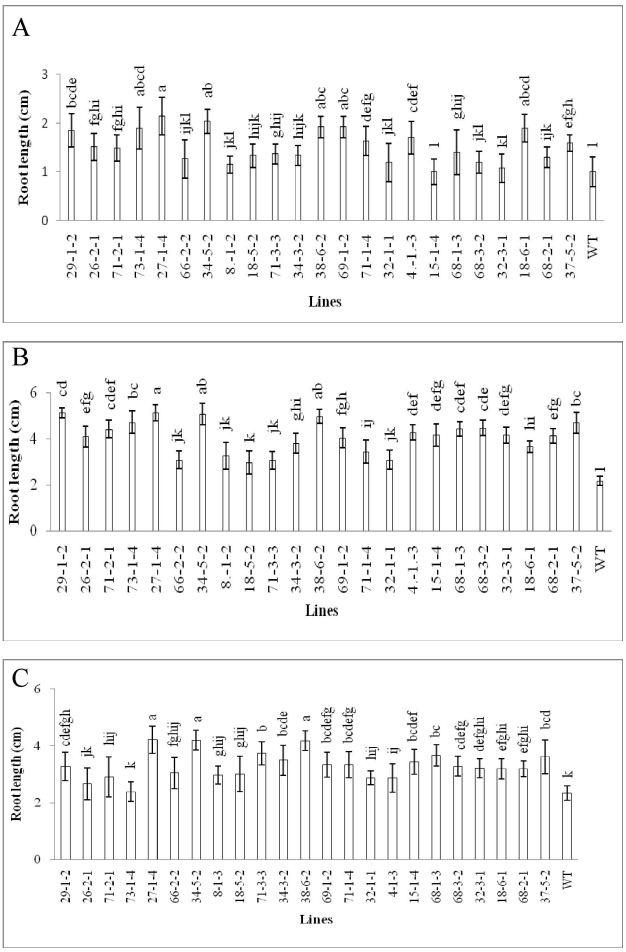

Fig. 1 : Growth response of rice (AC39020) transgenic lines exposed to NaCl, PEG, and $CdCl_2$ stresses. Graph depicting the root length of wild type (WT) and transgenic lines (indicated by number) of rice subjected to gradual stress induced by NaCl (300 mM) (A), PEG 8000 (-8 bar) (B) and $CdCl_2$ (800 µM) (C), letter with lower case and common are not significantly different by DMRT test (*P>0.05*).

(+, -) and 4th quadrant (-, +). This type of analysis would provide an option to identify the plants which have highest value for select parameter amongst the whole lot of plants from the experiments (Ramu *et al*., 2012). As expected the genotypes/lines selected were grouped into 4 different quadrants and the genotypes belonging to quadrant 3, considered as superior types, were selected based on their comparative performance under different combinations of stresses as shown in Fig. 2. The transgenic lines in quadrant 3 showed significantly higher growth compared to those that fall under quadrant 1; this trend was similar in all types of abiotic stresses. From the 23 lines tested, three lines *viz.,* 27-1-4 (L-1), 34-5-2 (L- 2) and 38-6-2 (L- 3) were identified as tolerant types and line numbers 8-1-2 (L-4), 15-1-4 (L-5), 32-3-1 (L-6), 18-5-2 (L-7), 71-3-3 (L-8), 26-2-1 (L-9), 71-2-1 (L-10) and 73-1-4 (L-11) as the lines with least cellular tolerance capacity under different stress conditions.

To validate the approach used, seedlings from contrasting lines were exposed to NaCl-, PEG- and $CdCl_2$-induced stresses and growth responses were analyzed. A higher per cent change in growth over the wild type (poor cellular tolerant type) under the different stresses indicated superior cellular tolerance in the selected tolerant transgenic lines (Fig. 3). Further, to corroborate the protocol developed, the selected superior transgenic lines were tested against wild type, under osmotic stress. Mannitol (200 mM) induced stress was imposed to compared the cellular tolerance capacity of the transgenic lines (superior type) with the non-transgenic (wild-type, poor type) plants. As shown in the Fig. 6, the select lines exhibited better growth over the wild type.

To confirm the transgenic nature of the superior lines, the presence of marker gene, *PsAKR*1, was examined by PCR-based approach. The integration of the marker transgene was confirmed as evidenced by the amplicon size of 583 bp (Fig. 4). Expression of the integrated marker gene was tested by semi-quantitative RT-PCR of *PsAKR*1 (Fig. 5) and also by glyphosate tolerance assay, as the marker gene, *PsAKR*1, imparts tolerance to the herbicide. For glyphosate tolerance assay, the rice seedlings from the select lines were grown under greenhouse conditions and the healthy newly emerged third leaf from top was selected to swab the glyphosate (750 ppm). All the selected lines showed tolerance to the herbicide while the non-transgenic wild type plant exhibited a sensitive phenotype.

In this study, a robust protocol to screen the plant genotypes for cellular tolerance at seedling stage has been developed and demonstrated. There are different methods reported to identify positive transgenic rice lines. Most of them include approaches such as

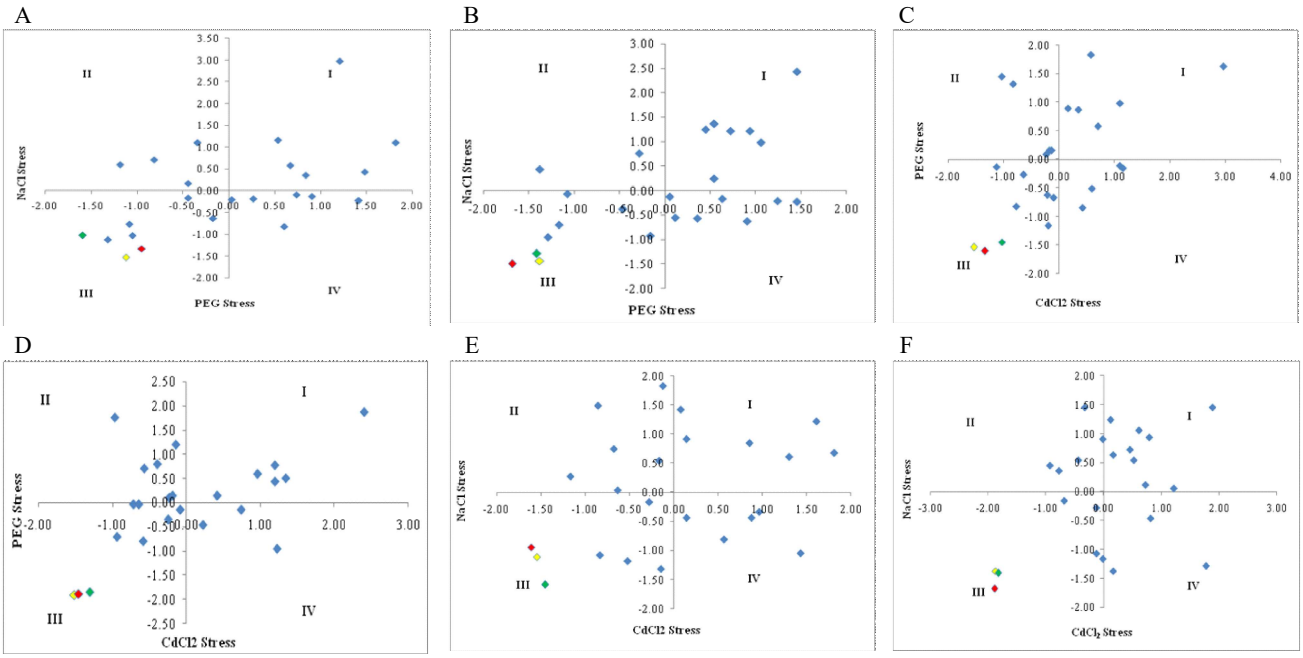

Fig. 2 : Performance of rice (AC39020) transgenic lines exposed to NaCl (300 mM), PEG 8000 (-8 bar) and $CdCl_2$ (800 iM) induced stresses. Z-scatter diagram depicting the comparative performance under two different stress conditions in terms of shoot length (A), (C), (E) and root length (B), (D), (F).

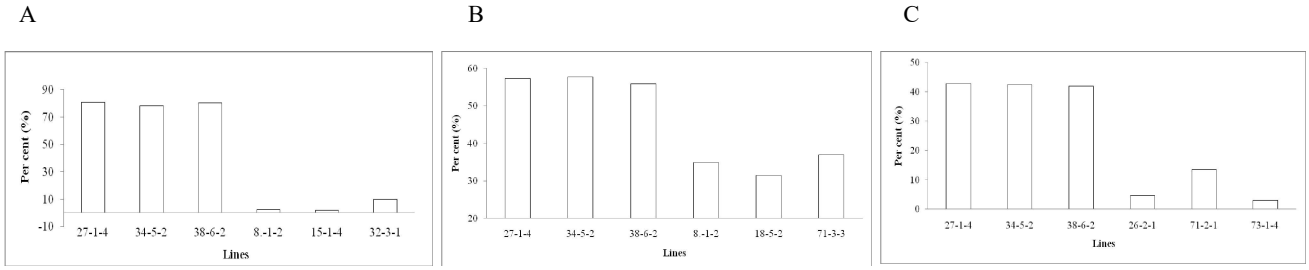

Fig. 3 : Comparative response of rice select transgenic lines exposed to NaCl (300 mM), PEG 8000 (-8 bar), and $CdCl_2$ (800 µM) induced stresses. Graph depicting the percent change in the shoot and root length of rice transgenic lines over the wild type under NaCl (A) PEG (B) and $CdCl_2$ (C) stresses.

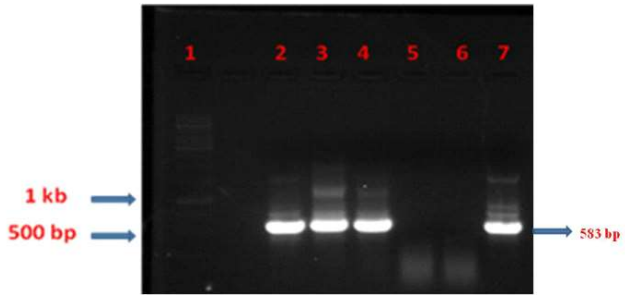

Fig. 4: Ethedium bromide stained agarose gel showing PCR product of select transgenic genes. Genomic DNA was isolated from transgenic plants and PCR was performed with *PsAKR*1 specific forward and reverse primers of marker gene, *PsAKR*1 (1:Ladder, 2:27-1-4, 3:34-5-2, 4:38-6-2, 5:WT, 6:Blank, 7:Plasmid).

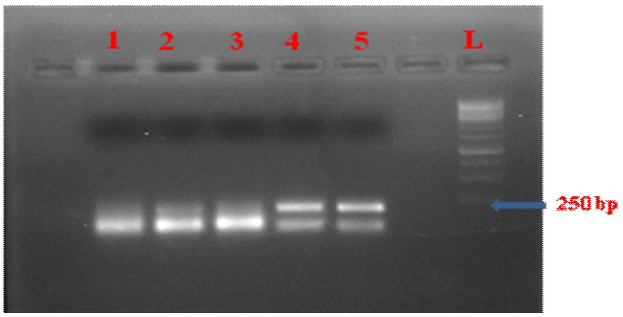

Fig. 5: Ethedium bromide stained agarose gel showing expression of PsAKR1 gene. 1: Blank, 2: WT, 3:27-1-4, 4:34-5-2, 5:38-6-2, L:1 kb gene ladder

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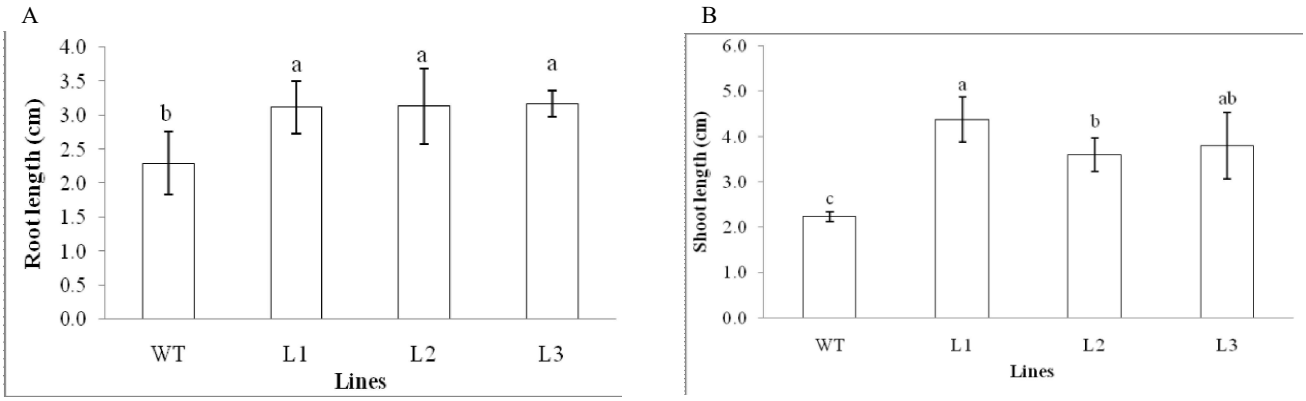

Fig. 6: Shoot (A), and root (B) length (cm) of seedling of wild type (WT) and select transgenic lines (L1:27-1-4, L2:34-5-2, L3:38-6-2) exposed to mannitol (200 mM) induced osmotic stress, letter with lower case and common are not significantly different by DMRT test (*P>0.05*)

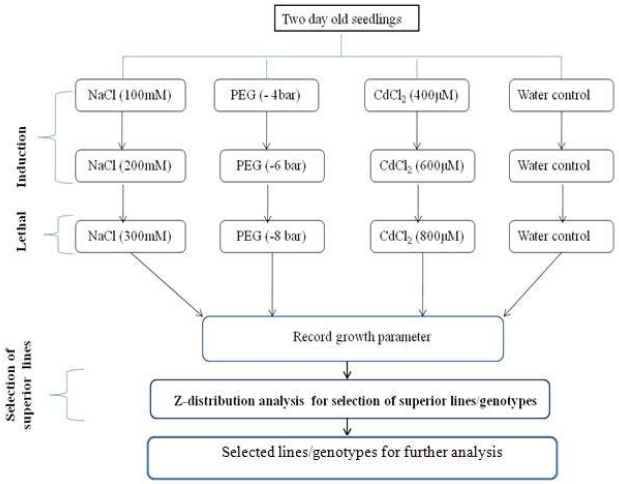

Fig.7 : Proposed screening protocol for identifying plant genotypes with superior cellular tolerance

involving simple methods like PCR alone (Mayer *et al*., 1992) or by screening approaches using marker gene/transgene activity (Noor *et al*., 2000 and Tan *et al*., 2005) or both the approaches (Dong *et al*., 2017). However, the robustness of the screening approach vests in the capacity to simultaneously identify a positive transformant which is also superior with respect to its performance. In this study, a potent strategy with multi-stress screening approach has been demonstrated, which would be useful to identify plant types exhibiting superior stress tolerance by virtue of their cellular tolerance capacities. The approach used was robust and can be easily adopted to screen large numbers of transgenic plants/germplasm accessions for superior cellular tolerance mechanism(s). Cellular tolerance is a complex trait with multiple associated pathways which is difficult to phenotype; therefore, the approach demonstrated in this study will be highly beneficial to select plants with superior stress tolerance capacities. Based on this study, a protocol to screen transgenic lines/germplasm accessions with enhanced abiotic stress tolerance potential, by multiple stress induction approach has been proposed (Fig.7). The approach proposed in the present study can be modified by employing different abiotic stresses/other related stress challenges depending upon the crop type or purpose.

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