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OPINION PAPER

Taking transgenic rice drought screening to the field

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Abstract

Numerous transgenes have been reported to increase rice drought resistance, mostly in small-scale experiments under vegetative-stage drought stress, but few studies have included grain yield or field evaluations. Different definitions of drought resistance are currently in use for field-based and laboratory evaluations of transgenics, the former emphasizing plant responses that may not be linked to yield under drought. Although those fundamental studies use efficient protocols to uncover and validate gene functions, screening conditions differ greatly from field drought environments where the onset of drought stress symptoms is slow (2–3 weeks). Simplified screening methods, including severely stressed survival studies, are therefore not likely to identify transgenic events with better yield performance under drought in the target environment. As biosafety regulations are becoming established to allow field trials in some rice-producing countries, there is a need to develop relevant screening procedures that scale from preliminary event selection to greenhouse and field trials. Multilocation testing in a range of drought environments may reveal that different transgenes are necessary for different types of drought-prone field conditions. We describe here a pipeline to improve the selection efficiency and reproducibility of results across drought treatments and test the potential of transgenic rice for the development of drought-resistant material for agricultural purposes.

Key words: Drought, field, methodology, rice, screening, transgenic.

From the lab to the field: a reality check

Despite many transgenic studies reporting candidate drought resistance genes and mechanisms (Bajaj *et al*., 1999; Umezawa *et al*., 2006; Shinozaki and Yamaguchi-Shinozaki, 2007; Bhatnagar-Mathur *et al*., 2008; Nakashima *et al*., 2009; Hirayama and Shinozaki, 2010; Pardo, 2010; Yang *et al*., 2010), no transgenic rice with improved performance under drought is currently in the pipeline for approval to our knowledge. Most transgenic rice drought studies have been performed under controlled conditions at early growth stages, and only a limited number of studies have demonstrated effects on yield under drought in field conditions (Du *et al*., 2010; Hu *et al*., 2006; Huang *et al*., 2007; Islam *et al*., 2009; Jeong *et al*., 2010; Oh *et al*., 2009; Xiao *et al*., 2009; Xiao *et al*., 2007). The use of unrealistic dry-down conditions in the majority of transgenic studies will limit the chances of selecting lead events applicable to the target environment. Is there a gap between knowledge of gene networks in the laboratory and development of drought resistant transgenic events? In conventional rice breeding, a breakthrough in the development of drought resistant lines was achieved during the last decade when direct selection for yield under both drought stress and well-watered conditions was employed (Atlin, 2003; Kumar *et al*., 2008). Learning from this experience, we argue that part of the solution for transgenics is to develop effective drought-screening protocols and increase the use of confined field trials in rice-growing countries to select for grain yield under drought stresses that are relevant for agricultural productivity.

Reports of drought resistance using transgenic technology from controlled environments are increasing. Rice is commonly used in transgenic drought studies as it is one of the most important food crops (Hirayama and Shinozaki, 2010; Deikman *et al*., 2011). Regardless of the screening procedure, experiments using genetic engineering have all tested the hypothesis that an inserted

transgene would confer improved resistance to drought compared with the receiving genotype (wild type) or untransformed null segregants (azygous). Out of 64 experiments published in the recent literature reporting better rice performance under drought using genetic engineering (since 2000, PubMed Central and Web of Knowledge), only 18% have reported grain yield data. A majority of the studies on transgenic rice have primarily aimed at identifying candidate genes, validating genetic pathways, and analysing the molecular and physiological functions of the transgene rather than demonstrating positive effects under realistic drought conditions. Two-thirds of the studies reported molecular and physiological experiments conducted at the vegetative stage using hydroponics, excised leaf dehydration, small pots, or trays simulating paddy dry-down conditions (Fig. 1A). Although those fundamental studies have been efficient in identifying genes, modes of gene action, and in uncovering gene networks, results obtained on drought resistance *per se* might not be reproducible in mature plants under field drought conditions.

The uncertainty about translating drought resistance from lab results to the field is mostly due to the complexities of natural drought events and plant responses to multiple environmental signals. The plant responses and traits involved to escape, avoid, and/or tolerate drought stress vary according to the developmental stages at which the stress occurs, level of drought severity, and the field environment (Pantuwan *et al*., 2002; Lafitte *et al*., 2007; Kamoshita *et al*., 2008). In an agricultural context, drought can occur simultaneously with other abiotic stresses such as high temperature (Pantuwan *et al*., 2002; Mittler, 2006). Those environmental effects often cannot be mimicked or detected in pots and artificial substrate-based studies (Mittler, 2006; Hervé and Serraj, 2009; Salekdeh *et al*., 2009; Mittler and Blumwald, 2010; Pardo, 2010; Deikman *et al*., 2011; Peleg *et al*., 2011; Varshney *et al*., 2011). Since transgenic events that would be useful for agriculture are expected to perform well under a range of environmental conditions, evaluation of transgenics in field or field-like conditions is essential.

Biosafety regulations and the costs associated with large-scale transgenic studies are likely to have limited many public research efforts to evaluate transgenic rice events in fields. Restrictions on material exchange and screening sites have also impaired

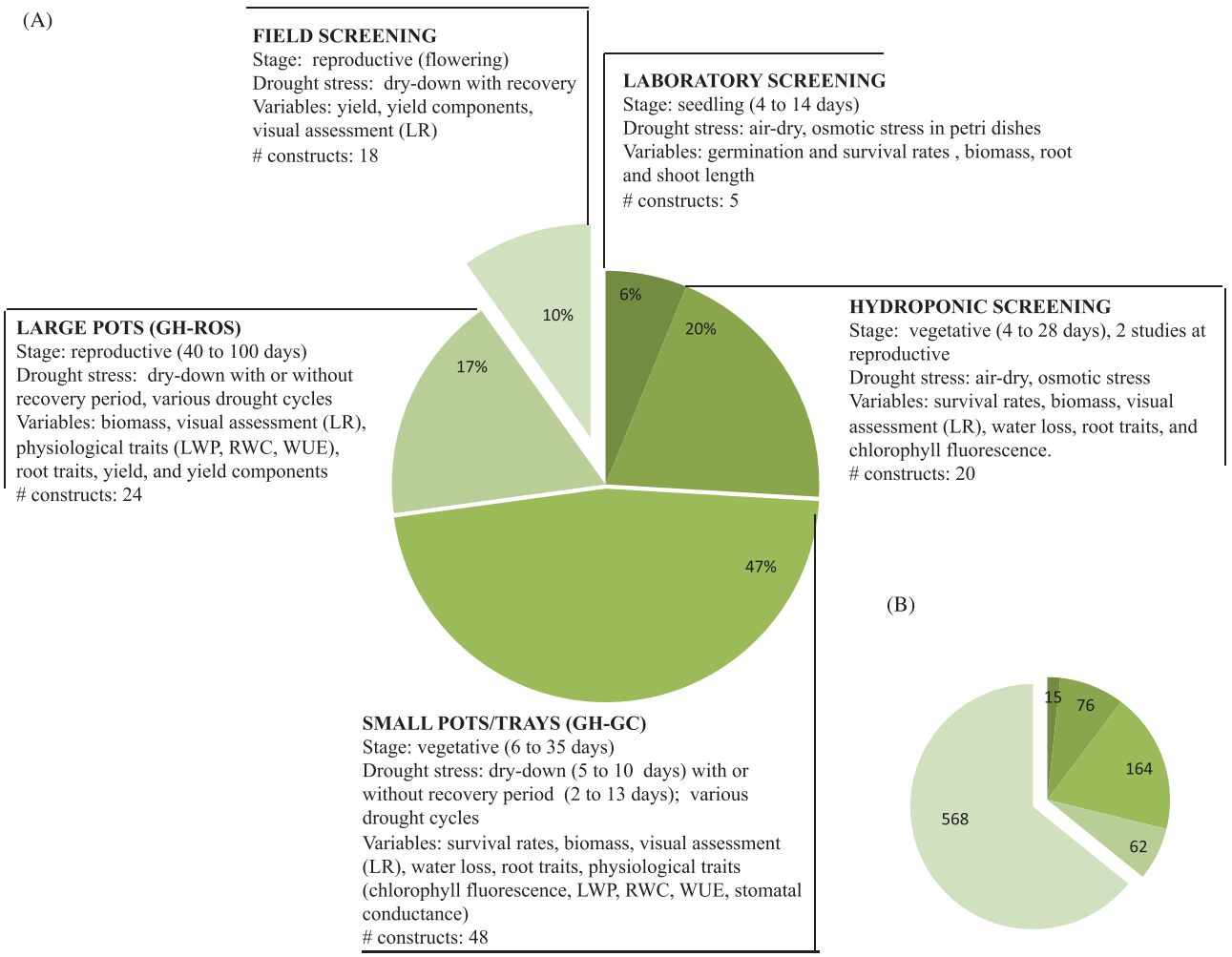


Fig. 1. Reported methods used for transgenic rice screening for drought tolerance. Data were collected from 64 publications from 2000 to 2011 reporting increased drought tolerance due to transgene expression. (A) Percentages of studies using one or various methodologies. (B) Number of independent events screened per methodology. GC, growth chamber; GH, greenhouse; LR, leaf rolling; LWP, leaf water potential; ROS, rain-out shelter; RWC, relative water content; WUE, water-use efficiency.

the scale at which transgenic events are evaluated in confined field environments. In light of those restrictions inherent to transgenic technology, screening large numbers of transgenic lines at the scale of conventional breeding programmes in various target local environments is challenging (Xiao *et al.*, 2009; Serraj *et al.*, 2011). In maize, mostly evaluated in North America where biosafety regulations facilitate transgenic field tests, event MON 87460 developed by the private sector has recently been deregulated in the USA as the first transgenic drought-tolerant crop (Animal and Plant Health Inspection Services, 2011). For rice, biosafety policies for multiple confined field evaluation are becoming increasingly established and are currently functional in some rice-growing countries, including India, China, the Philippines, Indonesia, and Bangladesh in Asia, South Africa and Burkina Faso in Africa, and Colombia, Argentina, and Brazil in South America (Fig. 2) (Adenle, 2011). This trend opens the prospects for development of large-scale screening facilities in regions where rice is bred and grown. Since numerous drought-resistance transgenes previously identified in laboratory or greenhouse studies are awaiting yield evaluation under drought conditions relevant to agricultural production, building a network of transgenic field-screening facilities will be beneficial and timely.

Previously reported transgenic-screening procedures

A wide array of methods has been reported in transgenic drought studies, ranging from small-scale desiccation or osmolyte studies to dry-down studies in pots or fields. Small-scale studies allow rapid visual screening of a large number of events in a homogeneous controlled environment ideal for molecular work. These experiments imposing rapid, severe, and continuous drought stress at the seedling/vegetative stage might help identify transgenic genotypes adapted to early stress occurring after direct seeding or in lowland nurseries. However, evaluation under reproductive-stage drought stress is recommended for applicability to agriculture (Atlin, 2003) as the majority of upland and lowland rice plants in rain-fed and intermittently irrigated areas experience terminal stress, with periodic drought between rainfall events at later developmental stages (Kamoshita *et al.*, 2008).

Secondary traits are mostly used when screening at earlier stages; however, grain yield under drought stress should be the primary trait used to select transgenic events in field or large pot studies. Indirect measures of drought resistance, such as survival rates and leaf rolling under stress, are the most popular screening variables in rice transgenic studies (Fig. 1A). Those two variables

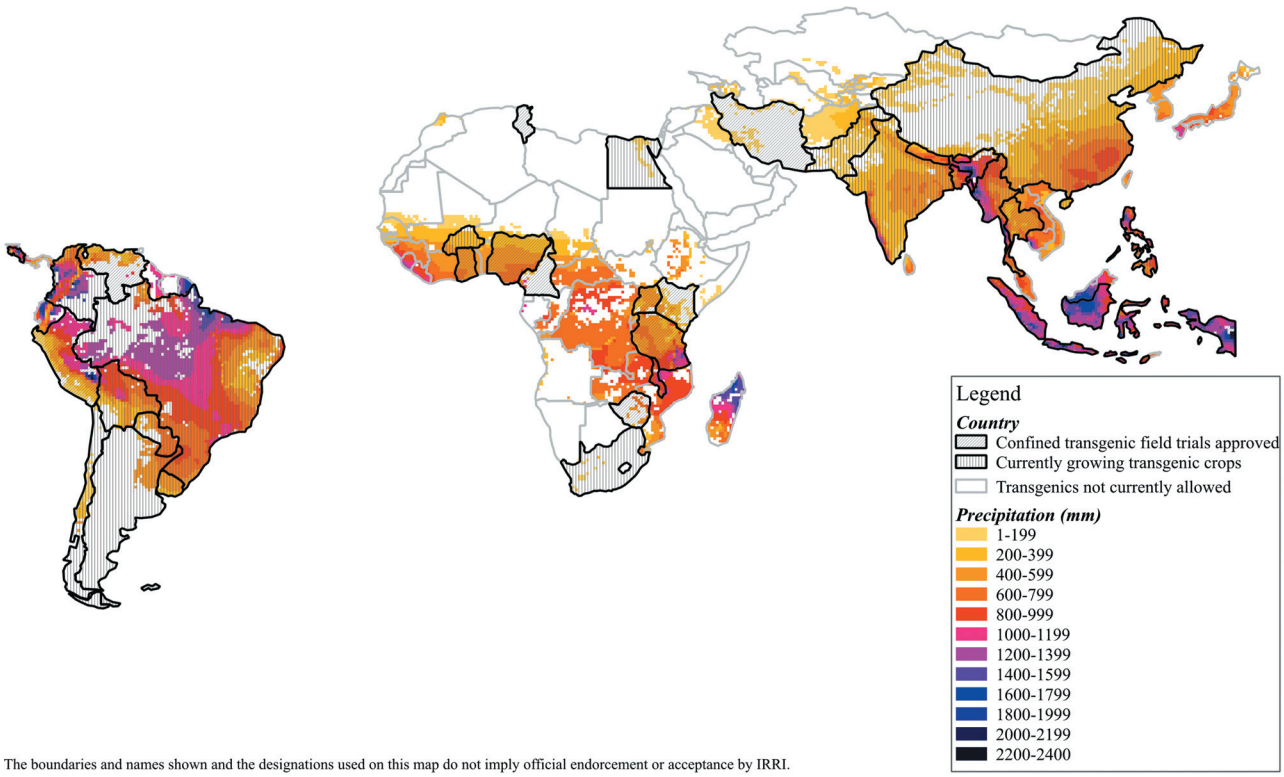


Fig. 2. Potential for transgenic evaluation in drought-prone rice-growing areas. Average cumulative precipitation (1960–2002) for the peak rice-growing season in rice areas of Asia, Africa, and South America is presented (University of East Anglia Climatic Research Unit, 2002). Non-rice-growing areas were removed using Monthly Irrigated and Rainfed Crop Areas (MIRCA 2000, Portmann *et al.*, 2010). Rice growing seasons were determined by using the IRRI rice map (IRRI, 2012 Sub-national Rice Crop Calendar) selecting areas for the peak of the main growing season from the month of establishment until 150 days after the first possible date of establishment. Countries currently growing transgenics or with trial fields approved were based on International Service for the Acquisition of Agri-biotech Applications reports (James, 2011).

were recorded in 48% of the transgenic studies, and 25% of those studies evaluated drought resistance solely based on those traits. To be useful measures of drought resistance, secondary traits must correlate with grain yield in the target environment (Blum, 2005). However, these leaf traits are not always correlated with yield under stress (Mitchell *et al*., 1998; Lafitte, 2003) and transgenic events that show drought resistance based on seedling survival to extreme drought stress may show low ability to recover and poor grain yield at maturity (Hervé and Serraj, 2009; Serraj *et al*., 2009). Rice is thought to achieve adaptation to drought mainly by dehydration avoidance and escape rather than desiccation tolerance (Blum, 2011). Traits related to dehydration avoidance (e.g. root or other traits maintaining leaf water status), escape (e.g. flowering date), or recovery (e.g. green canopy cover, tillering ability) offer better scope for selecting drought-tolerant genotypes for rain-fed environments (Lafitte and Courtois, 2001; Lafitte *et al*., 2007; Kamoshita *et al*., 2008) but are difficult to measure in small early growth-stage experiments.

Field studies in drought-prone areas or under rain-out shelters are represented in only 10% of transgenic rice screening studies in the literature (Fig. 1A). Reported field trials have been used for the evaluation of larger homozygous populations (on average 15 independent events per construct) with severe and moderate drought treatments imposed at the reproductive stage, followed by rewatering for a recovery period after pollination. Although the number of transgenic field studies in the literature is small, similar numbers of constructs have been screened in field studies compared to hydroponic studies. Three-times more events have been evaluated in the reported field studies compared to any other method (Fig. 1B). This comparison points to the enhanced capacity of field and rain-out shelter trials to screen large numbers of events per construct and increase throughput of transgenic evaluation for drought tolerance.

In addition to the variation in screening methods and traits used to quantify drought resistance, event selection criteria (including the use of homozygous events) and the number of independent events tested have also varied. Soil moisture levels and drought stress severities are seldom reported. Therefore, establishment of procedures setting basic guidelines for drought screening in the target environments could allow better comparisons of tolerance levels across genetic constructs and more accurate identification of lead transgenic events for further field evaluation.

At the International Rice Research Institute (IRRI, Los Baños, Philippines), in collaboration with other partners, we have developed more than 3500 transgenic events and screened more than 600 single-copy fertile independent events for drought resistance from a total of 19 gene constructs over 6 years. This research has been conducted in paddy-like screenhouses and in field experiments, in compliance with the biosafety regulations in the Philippines. Although the screening strategy must be adapted to country-based regulations and rice ecosystems, we suggest a framework to guide researchers during the early stages of development and screening of transgenic lowland rice lines (Fig. 3). This workflow was optimized to (1) ensure reliable and robust measurements of the transgene effects, (2) assist the transition of a large number of transgenic events from laboratories to fields early in the screening procedure, (3) effectively evaluate transgenic rice events for drought tolerance in an agricultural context, and (4) combine evaluation for yield increase under realistic drought conditions with functional transgene characterization.

Preselection of transgenic events

Developing transgenic material includes various rounds of molecular selection and genotyping prior to the evaluation of drought resistance. Therefore, field evaluation of grain yield in transgenic rice under drought requires a well-coordinated workflow between genotyping and field schedules. To accurately measure the effects of a transgene on yield under drought stress, it is essential to first identify homozygous events with single copy of the transgene. Null azygous segregants should be included as check lines during the screening process to verify that any drought effect observed is due to the presence of the transgene, rather than somaclonal variation from the regeneration process. Although transformation protocols are typically optimized to minimize the risk of chromosome rearrangement and epigenetic variation during transformation, it is necessary to evaluate nulls to control for somaclonal drag.

After transformation, positive events for the transgenes should be first selected for single-copy insertion, fertility, normal phenotypic appearance to omit off-type plants, and optionally transgene expression in T0 plants. Off-type plants and low fertility can be caused by somaclonal drag or overexpression when using constructs with strong constitutive promoters, particularly in combination with transcription factors (Dubouzet *et al*., 2003; Nakashima *et al*., 2007). Screening of 10–20 single-copy T1 events per construct is recommended to confirm the transgene effect under drought and to account for variability in gene positional regulation (Hervé and Serraj, 2009; Xiao *et al*., 2009).

At the gene validation stage, when a large number of constructs are evaluated, positive T1 transgenic events and azygous null segregants are selected by screening for a selectable marker and for the transgene. Prior to transplanting, a large number (in excess of the planned number of transgenics for the experiment) of T1 events are typically sown in seedling trays and grown for 14–20 days. During growth in trays, leaves are painted with a selectable agent and scored for lesions, which appear only on untransformed plants. This allows selection of a subset of positive events for further genotyping by PCR and negative events for use as azygous nulls. The most commonly used selectable marker agents in rice transformation are hygromycin and an herbicide containing phosphinotricin (Twyman *et al*., 2002). Leaf painting can be carried out using either selectable agent. Homozygous lines for each individual event can be identified in T2 seeds based on Mendelian segregation ratios for resistance to the selectable agent in Petri plates or by PCR (Fig. 3).

Transcript analysis at early stages in the pipeline is optionally carried out to avoid advancement of events with silenced transgenes to subsequent screenings. If a drought-inducible promoter is used, detection of silenced events can be based on semi-quantitative transcript expression in a cut leaf exposed to dehydration for few hours. If space is not a limiting factor, direct phenotyping of a large number of single-insert T1 plants may be favoured to increase the throughput and scale of the screening without a larger investment in laboratory supplies. Gene

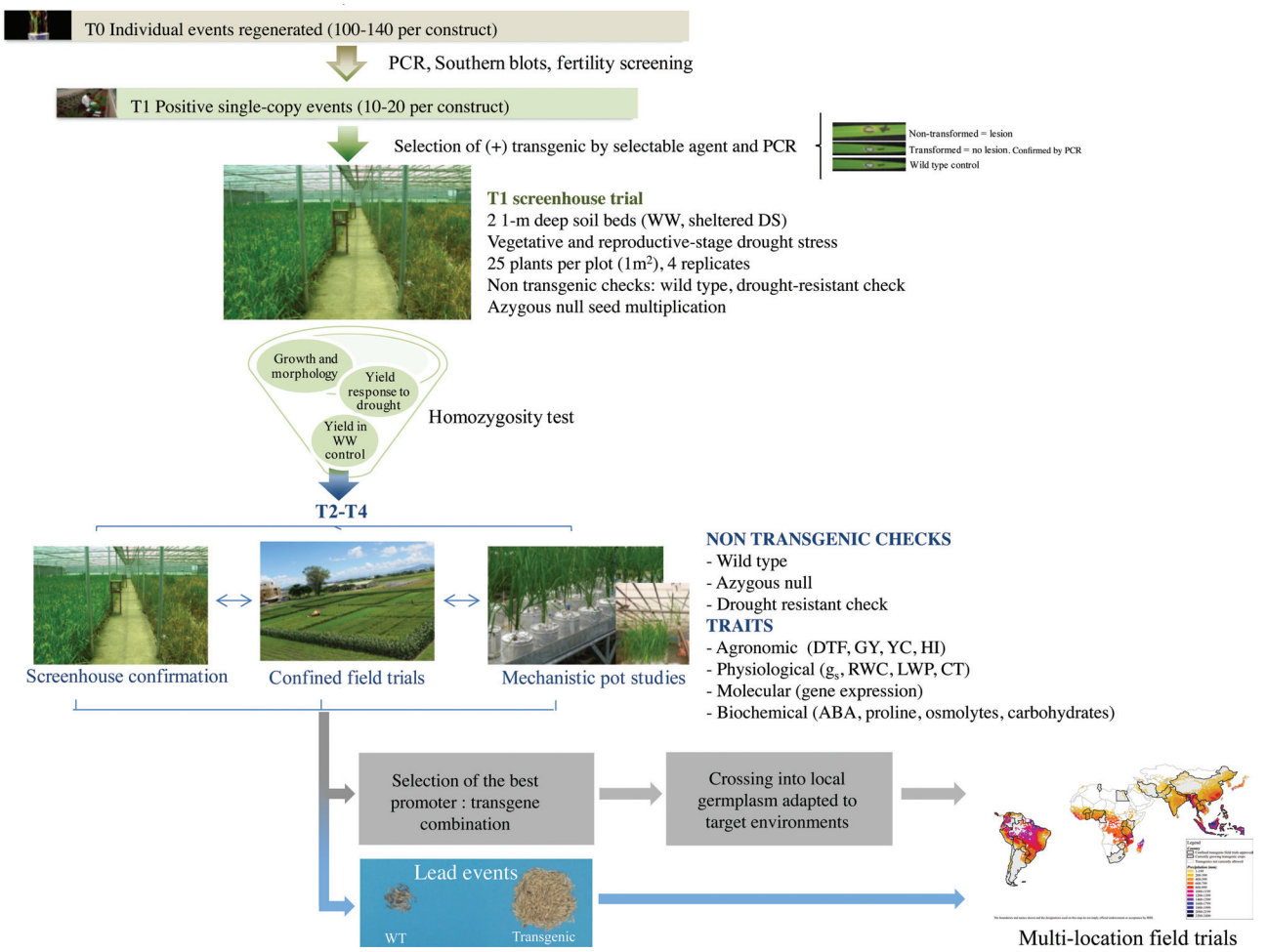


Fig. 3. Screening procedure for lowland transgenic rice evaluation under drought stress at IRRI. After transformation, positive events for the transgenes are regenerated and genotyped to select single-copy T0 positive events with good fertility rates and no abnormal morphologies. Independent T1 events are grown and subjected to reproductive drought stress in a paddy-like screenhouse and evaluated for yield under drought stress and well-watered conditions compared with the wild type and drought-tolerant check. Promising homozygous T2 events are then compared with the azygous control as well as wild type in field trials, screenhouse, and pot studies. Measurement of physiological, molecular, and biochemical responses in subsequent screening is performed to identify secondary traits associated with transgene expression and elucidate the basis of tolerance observed in the field or screenhouse. ABA, abscisic acid; CT, canopy temperature; DS, drought stress; DTF, days to flowering; g_s, stomatal conductance; GY, grain yield; HI, Harvest index; LWP, leaf water potential; RWC, relative water content; WT, wild type; WW, well-watered; YC, yield components.

expression analyses can then be performed further along in the pipeline on a promising subset of homozygous T2 events to investigate correlations between genotype, gene expression, and the phenotype under stress.

Getting closer to real drought scenarios: screening approaches to evaluate transgenic rice under drought

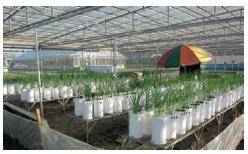
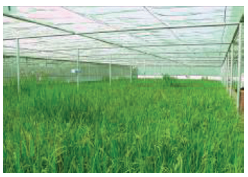
Aside from the complexities and costs associated with evaluation of transgenics, the main difference between conventional and transgenic field evaluations is the requirement to conduct the experiments in confined environments. This limits the amount of germplasm that can be screened and the opportunities for multilocation trials. The variability in drought environments and the combination of stresses to which the transformed events are subjected may have a large influence on the expression of the transgene, especially if stress-inducible promoters are used. If carefully monitored, environmentally induced differences in drought timing and intensities among experiments can be used to link transgene effectiveness across a range of drought stresses, thus taking full advantage of the unpredictable experimental conditions inherent to drought field treatments. However, the mechanisms and traits involved in response to vegetative-stage drought stress may be different than those important in reproductive-stage drought resistance. They may also vary with drought intensity and other abiotic stresses occurring simultaneously. These complexities question the possibility of a single transgene conferring consistent yield advantages under a range of drought environments (Sinclair and Muchow, 2001; Pantuwan *et al*., 2002; Sinclair *et al*., 2004; Lafitte *et al*., 2007; Kamoshita *et al*., 2008;

Guan *et al.*, 2010) and different sets of genes may be necessary for each drought scenario.

Screening under drought stress with a gradual onset and moderate-to-severe drought at targeted growth stages is recommended to reflect drought in typical rain-fed lowland cropping environments and to evaluate if the transgene effect is robust. Several rice drought improvement programmes are currently in agreement with the effectiveness of direct selection for yield under drought, rather than for secondary traits (Atlin, 2003; Venuprasad *et al.*, 2007; Kumar *et al.*, 2008). In our approach, standardized protocols used to screen conventional germplasm and apply stress at reproductive stage during the dry season have been adapted to evaluate transgenics for grain yield under realistic drought conditions (Fig. 3). Field screening of transgenic rice yield under drought from the National Center of Plant Gene Research in Wuhan, China (Hu *et al.*, 2006; Xiao *et al.*, 2009) and from Myongji University, Korea (Oh *et al.*, 2009; Jeong *et al.*, 2010), have included the use of moveable rain-out shelters for applications of drought stress at reproductive stage. Rain-out shelters are ideal for conducting experiments in climates where rainfall may disrupt the drought treatments. However, screening for yield in unsheltered field experiments under managed drought stress by screening the lines in the dry season has also been effective, as evidenced by the multiple major-effect drought-yield quantitative trait loci that have been identified in rice (Bernier *et al.*, 2009; Venuprasad *et al.*, 2009; Vikram *et al.*, 2011). Besides dry-season screening, another approach is to delay planting during the wet season to synchronize the reproductive phase of the crop with the period with minimal chances of rainfall after the end of monsoon rains. Plant performance comparing these two screening protocols (the dry season at IRRI and delayed in the wet season in India) have been reported to be highly correlated (Verulkar *et al.*, 2010).

Developing realistic dry-down conditions in experimental set ups that simulate lowland drought environments is essential to predict which transgenic events would be likely to show yield benefits in an agricultural context. At IRRI, this is accomplished using a combination of field and screenhouse experiments (Fig. 3, Table 1). A screenhouse is composed of two sides with independent, 1-m-deep intact soil profiles. One side of the screenhouse is equipped with a pump and drainage system to aid in removal of groundwater and is sheltered from above to exclude rainfall. The other side is open to rain, allowing the screening of events under drought and well-watered conditions simultaneously (Hervé and Serraj, 2009) (Table 1). Soil is flooded and puddled in both treatments and periods of managed drought stress are imposed in one side of the screenhouse by draining, with drought stress developing gradually after 3–4 weeks. Large (20 l) soil-filled pots are also used for physiological characterization while selecting for yield under drought in restricted confined environments. Drought stress in large pots can be more carefully managed compared to field conditions (Xiao *et al.*, 2007, 2009; Table 1) and can be controlled to impose a slow onset.

Table 1. Confined environments for evaluation of drought response and grain yield of lowland transgenic rice

	Dry-down characteristics	Advantages	Limitations
Greenhouse  Large pots allowing grain setting	Control of soil moisture by gravimetric measurements Managed dry-down Homogeneous drought conditions within pots and between events	Measurement of water uptake rate, transpiration, and water-use efficiency can be conducted Precise control of dry-down and rewatering using gravimetric measurements Specific growth stages can be targeted for each construct/event independently Allow comparing physiological responses among events at definite soil water content	Constrain root growth Poorly reflect field conditions Time and labour intensive Limited capacity in terms of the number of events that can be screened. Compliance to biosafety regulations for rice transformed with plant genes typically requires growth in a contained facility
Screenhouse  Deep paddy-like soil beds	Soil moisture levels are managed by draining the paddies, installing roofing, and irrigation Gradual dry-down	Paddy-like conditions but sheltered from rainfall Expression of avoidance traits (i.e. deeper root growth) is possible Experiments can be scheduled anytime of the year	Small plots Effect of roof and seasonal variations on dry-down dynamics, temperature, and radiation High disease and pest pressure Drought cannot be targeted to different growth stages within one experiment Compliance to biosafety regulations for transgenic rice transformed with plant genes typically requires growth in a contained facility
Field  Plots in drought-prone field	Soil moisture levels are managed by draining the paddies and irrigation during dry season Gradual dry-down Strong effect of location and year on drought conditions	High capacity: large number of events and constructs can be screened simultaneously Larger plots Experiment can be carried out in target environments Multiple drought treatments can be tested simultaneously (e.g. vegetative stage, reproductive stage, or both; with or without recovery)	Compliance to biosafety regulations requires stringent control and large field size (physical barrier, temporal and/or distance isolation, weed and volunteer management, separate equipment) Separate biosafety permit is required Drought timing and intensity depends on variations in rainfall, water table depth, and soil type

Soils in lowland rice systems are generally saturated before a drought period begins, taking from 2 to 3 weeks for stress to develop depending on soil texture and climate conditions. Since reproductive-stage drought stress is the most damaging to yield in rice (International Rice Research Institute, 1980), it should be applied during the first round of screening. The basic guideline for our transgenic-screening protocol – also used in the IRRI conventional drought-breeding programme – is to target drought stress during the reproductive stage by draining the field at panicle initiation, to rewater the experiment when the soil water potential at a depth of 30 cm reaches about –65 kPa (Torres *et al.*, 2012). It is recommended to include an advanced conventional drought-breeding line as a drought-tolerant check in transgenic studies. Vegetative-stage stress treatments are imposed as soon as plants are established after transplanting, followed by rewatering as necessary for the experimental objectives. An effective drought stress treatment should reduce mean yield by 65–85% compared with the fully irrigated control in order to identify drought-tolerant lines (Kumar *et al.*, 2008). The environment and soil properties should be well characterized and soil moisture content recorded during dry-down to describe the severity and timing of drought stress.

Potential for transgene evaluation in drought-prone rice-growing environments

Conducting a confined transgenic rice field trial requires long-term planning, involvement with local and national regulatory institutions, and compliance with national biosafety regulations. It is therefore important to select an appropriate drought-prone screening site located in target environments with historically low rainfall during the dry season and with a biosafety framework in place for transgenic testing in field trials (Fig. 2). Although the presence of national regulations does not guarantee approval at the local level, various rice-producing countries currently have biosafety regulations in place for transgenic field trials. After the best promoter/gene combinations and lead events for grain yield under drought are identified, yield stability of the transgenics could be evaluated at multiple locations with well-defined drought conditions (Fig. 3). Production of large numbers of events from the best construct is often carried out to fulfil regulatory requirements of having a clean inserts with no backbone plasmid sequences and, if necessary, marker-free inserts to ease public acceptance. Hygromycin phosphotransferase (*hph*) and other selectable marker genes have been deregulated (Center for Environmental Risk Assessment, 2010) and have a safe history of use (European Food Safety Authority Scientific Panel, 2004). Co-transformation or double T-DNA strategies to segregate out the marker gene or marker deletion via site-specific recombination may also be used (Hohn *et al.*, 2001; Vetten *et al.*, 2003).

Consideration of field experiments from an early start in a transgenic programme can facilitate targeting cultivars adapted to a specific drought-field environment, so that potential gains compared to local benchmark varieties can be estimated. For improved rice to be accepted by consumers, it is necessary to consider both adaptation to target environments and fulfilment of local grain quality and taste preferences. This is especially important in transgenic studies in which the recipient genetic background is often chosen according to its efficacy to be transformed rather than agronomic or cultural considerations. We recommended conducting the initial screening efforts in the genetic background of a mega-variety, since these are popular over large growing areas and locally adapted and because relatively quick introgression of the transgene into other mega-varieties is possible. Since deregulation of transgenics is generally event-based (Biosafety Clearing-House, http://bch.cbd.int/), further introgression in different genetic backgrounds through conventional and marker-assisted breeding is more efficient than reinitiation of genetic transformation.

Conclusions

Breeding for drought stress tolerance by directly selecting for yield under drought rather than for specific traits has met considerable success through screening in drought-prone fields. The current definition and measurement of drought resistance used in laboratory/greenhouse evaluations of transgenic material differs considerably from the definition of drought resistance at the agronomic scale. We argue that approaches toward the development of transgenic and conventional drought-tolerant varieties should be reconciled for transgenic technology to be considered as part of the solution to rice improvement for drought-prone environments. Biosafety regulations already in place in some rice-growing countries present opportunities to assess the effect of a single gene on drought resistance at an agronomic level across drought environments.

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