Chapter 2-3

Towards development of drought tolerant upland rice through international research collaborations from gene discovery to trait evaluation: in case of *OsNAC6* gene as example

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Abstract

Extreme weather events such as droughts and heat waves are likely to become more common in recent warmer climate. Thus, development of drought tolerant rice varieties has been an urgent research goal since rice is the staple food of many people on planet. To speed up development of drought tolerant rice varieties, unique research collaborations between advanced institutions and international research organization were developed and led by Japan International Research Center for Agricultural Sciences (JIRCAS). This collaboration brought diverse expertise together to facilitate a product development pipeline from gene to trait evaluation under target field environments. One of genes tested in this study was *OsNAC6* gene. Twenty-eight independent transgenic lines that possessed homozygous single copy of introduced *OsNAC6* gene were generated from a commercial tropical japonica variety, Curinga. Evaluation of the transgenic lines under controlled and confined rainfed conditions in Colombia revealed superior performance of some transgenic lines in grain yield compared with non-transgenic plants. The transgenic lines of other genetic backgrounds, NERICA1 and NERICA4, also showed increased grain yield under the rainfed conditions. The results strongly suggest that the gene works regardless of genetic background as common genetic component for drought tolerance in rice under field conditions leading to increased grain yield.

Key words: upland rice, trait evaluation, drought tolerance, transgenic approach

Introduction

Drought occur naturally but increased climate variability due to climate change accelerated to make it more extreme which increases risk of crop yield losses with many consequences. It was illustrated that globally, climate variability accounts for roughly a third of the observed yield variability, meaning that variations in temperature, rainfall or its combination explain yield variability (Ray et al. 2015). Thus, developing drought tolerant crop with minimum yield losses is crucial to meet the needs of future population growth in sustainable and environment friendly manner.

Plant's responses to water-deficit conditions have been extensively elucidated to identify drought inducible genes with various functions mainly in model plants such as Arabidopsis and a rice variety, Nipponbare (Shinozaki and Yamaguchi-Shinozaki 2007) and many of which showed increased drought tolerance under controlled conditions, meaning that most of candidate genes were still at "Gene/Trait Identification" and "Proof of Concept" (in this study demonstrating in principle with the aim of verifying gene function) phases in product development pipeline as indicated in **Fig. 1**. In this study, we moved forward from "Gene/Trait Identification" to "Early Development" and in some extent, "Advanced Development" phases to demonstrate 1) drought inducible genes work not only for drought tolerance but also for increased yield and 2) the genes work under real drought field conditions regardless of genetic background. Through research collaborations between advanced institutions and CGIAR centers (Gaudin et al. 2013), more than 15 genes and promoter combination have been evaluated among them, *OsNAC6* gene, a transcriptional activator up - regulating stress - inducible genes for stress tolerance was highlighted in the report since this gene showed clear evidence of better agronomic performance under drought conditions compared with other genes tested in this study.

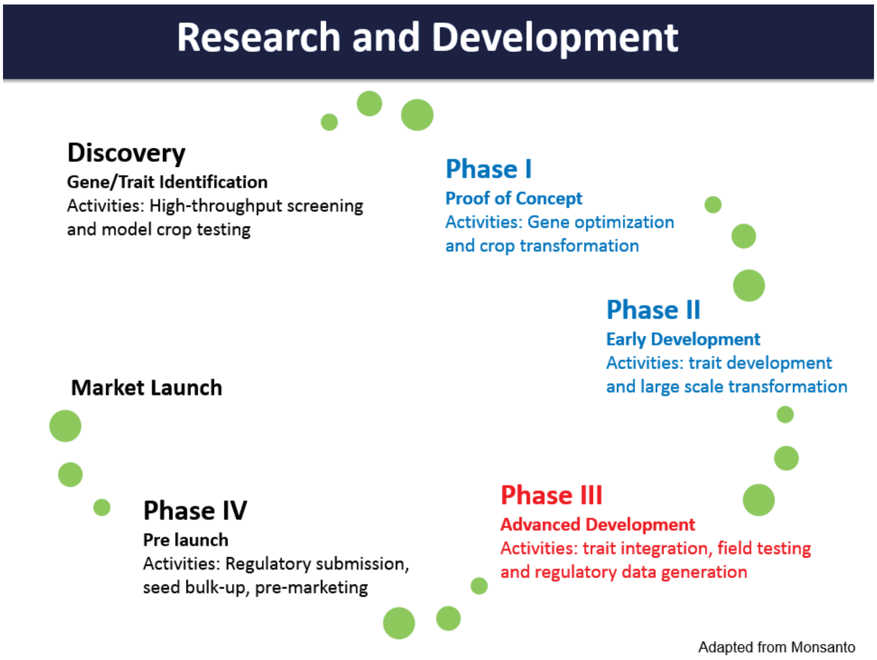

Fig. 1. Diagram of product development pipeline from Discovery to Market Launch.
Discovery and Proof of Concept work were done previously by RIKEN and JIRCAS for stress inducible genes and promoters used in for this project.

Materials and methods

QC procedure for gene constructs

Each construct provided by JIRCAS and RIKEN PSC was transformed into *Agrobacterium tumefaciens* strains EHA105 and *E. coli*, DH5. by electroporation. After gene confirmation by PCR using gene specific primers, the plasmid DNA of each construct extracted from *E. coli* DH5α was subject to sequencing for the region between T-DNA boarders except selection cassette.

Plant Material

Curinga, tropical Japonica upland rice, which showed better drought resistance compared with other varieties developed in Brazil (Breseghello et al. 2009) was used due to geological and economic importance of this genotype in Latin America, especially in Brazil when the project started in 2007. NERICA lines were produced by JIRCAS and used for agronomical performance under drought conditions at confined experimental fields managed by CIAT (Selvaraj et al. 2017). NERICA lines were reported most drought tolerant varieties among other varieties tested in water stress experiments in Uganda (Matsumoto et al. 2014).

Rice genetic transformation and isolation of transgenic events with low-copy number of the transgene

Each construct was transformed into *A. tumefaciens* (EHA105) and low-copied transgenic events are isolated as described in Selvaraj et al. (2017).

Drought trials under rainout shelter and rainfed conditions

At CIAT HQ, Palmira, two reproductive drought experiments per year were carried out one in rainy season (Feb-June) and another one in dry season (August-December) using rainout shelter in confined field. As to rainfed drought trials, confined field was established at Santa Rosa experimental fields and used to evaluate lines for agronomical performance in dry season starting in December. All the details of experimental design and agronomical analysis were described in Selvaraj et al. (2017).

Results and discussion

Isolation of single homozygous transgenic events

More than 15 constructs with gene and promoter combinations were received from RIKEN and JIRCAS in the period of 2007-2009 and one of which was pBIH-*osnac6::OsNAC6*. The *OsNAC6* was isolated as a transcriptional activator and up﹣regulates stress﹣inducible genes including lipoxygenase and peroxidase for stress tolerance (Nakashima et al. 2007), suggesting an important role of the gene for improved drought tolerance in rice. QC-confirmed *OsNAC6* gene construct was transformed to Curinga to generate 112 independent events and further transgene copy analysis resulted in 28 single-copy events at T_3 generations.

Establishment of confined fields for trait evaluation under drought conditions

To evaluate transgenic lines harboring gene of interest under real field drought conditions, two confined fields were prepared with permission of Colombia government. One was at CIAT-HQ under rainout shelter conditions and another was Santa Rosa experimental field where rainfall is limited from December to March during dry season. Permit of field testing of genetically modified rice for research purpose at these sites was obtained in 2008 and 2010, respectively as described in **Fig. 2**.

During the project period, CIAT established standard operating procedures to better manage transgenic materials from laboratory to field. On 2013, CIAT was certified as a member by Excellence Through Stewardship, a global not-for-profit organization that promotes quality management systems for agricultural technology products (https://www.excellencethroughstewardship.org/).

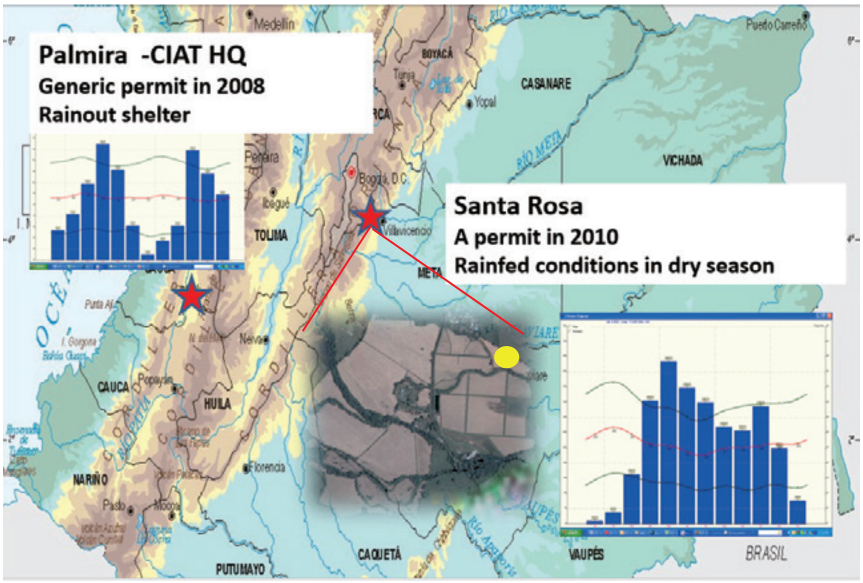

Fig. 2. Location of confined fields in Palmira and Santa Rosa and its rainfall pattern.
Star mark indicated geological location of Palmira and Santa Rosa in Colombia. Yellow dot indicated location of confined filed (40 m x 60 m) established at Santa Rosa experimental field station. Detail rainfall pattern in Colombia can be obtained at following site: https://en.climate-data.org/.

Evaluation of agronomical performance under reproductive stage drought stress conditions using rainout shelter

At first transgenic lines showing less yield and/or different plant type compared with parent genotype under well-irrigated conditions were eliminated during seed multiplication. Selected lines were further tested under reproductive stage drought stress conditions. The experiment was carried out with 52 transgenic events from Curinga and 18 transgenic events from NERICA representing 8 and 4 gene constructs respectively (data note shown). Drought was imposed by withholding irrigation when panicle initiation was around 10 mm long (63 days after sowing in the case of Curinga) for 3-4 weeks or until severe leaf rolling & drying appeared in non-transgenic control. Then the plants were re-irrigated to 90-100% field capacity till the physiological maturity. The intensity of drought was monitored through AquaPro soil moisture probes that could measure moisture in the soil profile of 0.85 m depth then plants were irrigated by boom to monitor the genetic variation in recovery from drought (**Fig. 3**).

After evaluation of agronomical performance under rainout shelter conditions, eight lines, namely, line 2967, 3008, 3012, 3074, 3080, 3085, 3270 and 3677 were selected (data not shown) based on better yield performance compared with non-transgenic plants and further tested at Santa Rosa experimental field for multiple years.

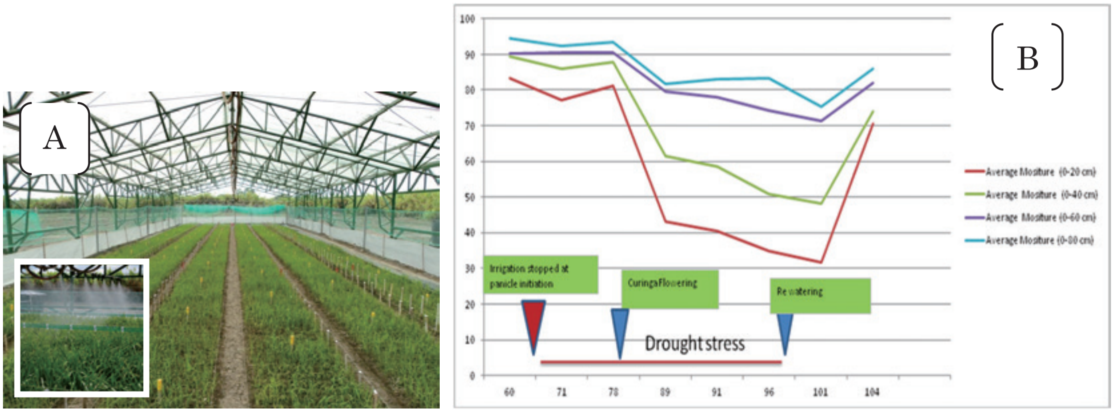

Fig. 3. Example of soil moisture profile during drought period in Feb-August 2012.
Rainout shelter at CIAT-Palmira was used for evaluation of Curinga transgenic lines under drought conditions at reproductive stage. [A] View of drought trial under rainout shelter. Photo in white boarder was boom irrigation. [B] Soil moisture profile with different depths from 20 cm to 80 cm. Three triangle markers indicated time of stopped irrigation, flowering time and re-watering.

Establishment of rainfed drought stress conditions for multiple year evaluation

Santa Rosa experimental field was used to establish the rainfed drought stress conditions. Initial field establishment was completed in 2010 after complying all the biosafety requirements (**Fig 4**). Under upland conditions, uniform seed germination was essential to establish a field trial. Therefore, initial irrigation was provided through sprinklers-irrigation till establishing the crop. During the field trial, soil moisture in different depths was monitored as indicator of severity of drought conditions.

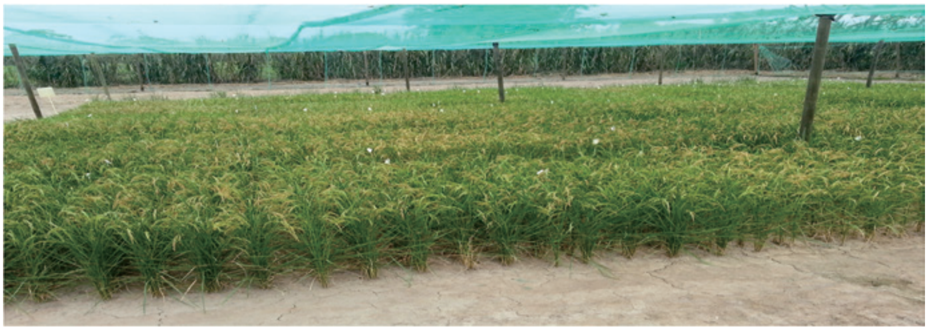

Fig. 4. A typical plot view under rainfed drought stress conditions at Santa Rosa experimental field.
Plot were covered by blue net to avoid rodents.

Fig. 5 was one example of field drought conditions from December, 2011 to March, 2012. In this trial, two dry spells were occurred, one at vegetative stage to initial panicle initiation stage (spanning about 36 days long) and one at late panicle initiation stage to flowering (19 days) (**Fig .5**). In between the two dry spells, at 54 days after sowing some rainfall around 40 mm was received to recover the drought stress. In the total crop period (sowing to harvest), plants received total of 336 mm of rainfall, it is little less against past ten-year average (388 mm). It's interesting to note that, more than 60% of the rainfall received after flowering (**Fig. 5**). The plants were under gone severe drought during late vegetative and panicle initiation stage.

Annual rainfall of this year 2011-2012 at this site is 2851 mm. Total rainfall during crop period (Dec-March) was around 336 mm. Average of maximum temperature was around 32ºC and relative humidity was around 85% during the crop period.

During vegetative stage upper layer (0-40 cm) moisture maintained around 60-68%, during the time of panicle initiation, moisture sharply declined up to 50%. The average soil moisture 0-80 cm was also declined below 60% during panicle initiation time (**Fig. 6**).

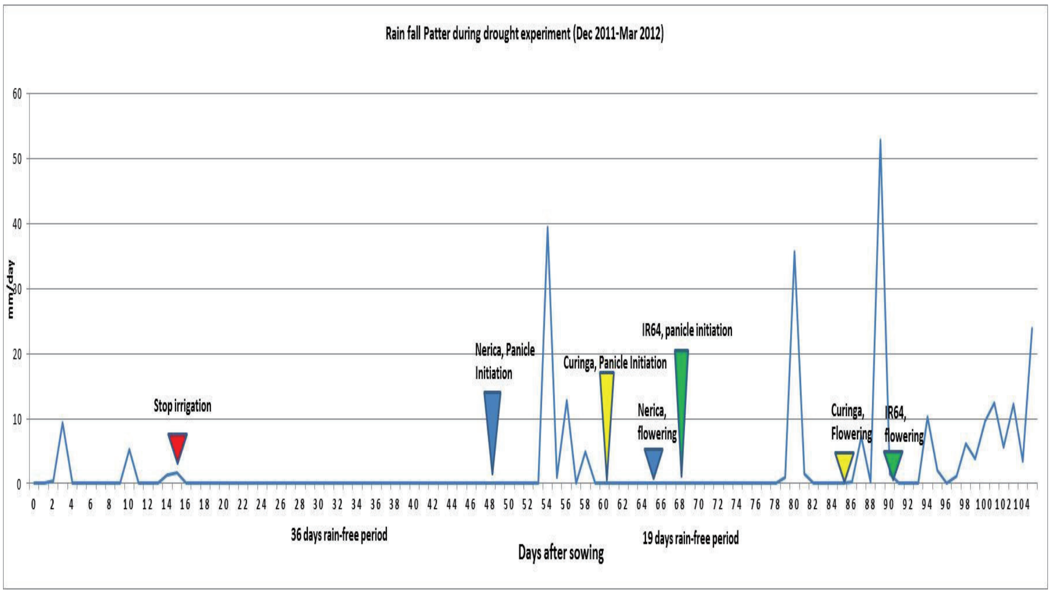

Fig. 5. Rainfall & dry spell pattern at Santa Rosa started from December, 2011.
Peak showed rainfall during the period of the experiment.

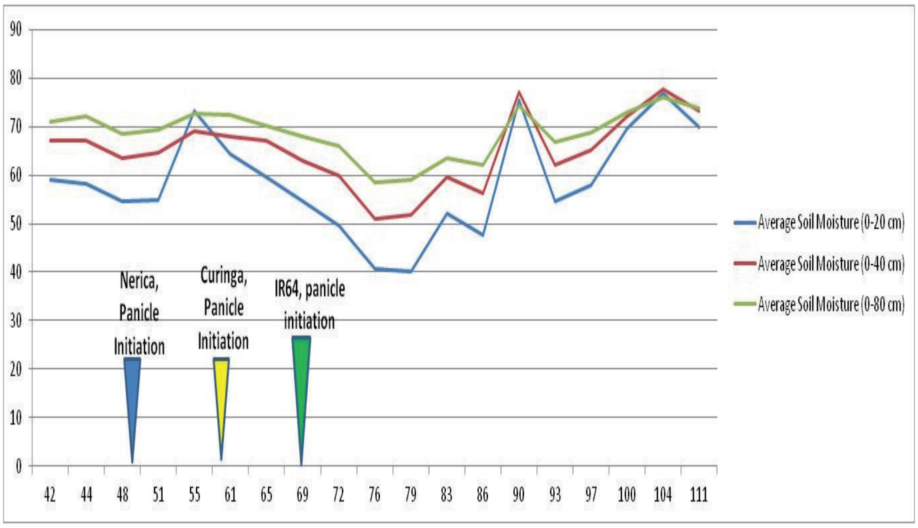

Fig. 6. Soil moisture profile during Santa Rosa rainfed trial during period of 2011-2012.
Purple line indicated moisture from 60-80 cm, Green line indicates 40-60 cm; red line indicates 20 - 40cm and blue line indicates 0-20 cm.

Third rainfed filed trial was initiated in December 2012 using eight promising lines from *osnac6::OsNAC6* as described above among other Curinga and NERICA lines (data not shown). This time the drought was very severe and 42 days rain free period was recorded and plants received most of the rainfall after flowering (data not shown). The grain yield was affected strongly among the transgenic lines. Among the 9 different constructs studied, namely *osnac6::OsNAC6*, *oshox24::OsNAC6*, *ubi::AtGolS2*, *oshox24::AREB1*, *oshox24::AREB2*, *lip9::DREB1C,* *osnac6::OsSCZF2*, *osnac6::AREB1ΔQT*, *lip9::AREB1ΔQT*, the positive effect of the constructs in terms of grain yield under stress was noticed including *osnac6::OsNAC6* and *Ubi::AtGolS2* (Selvaraj et al. 2017). The yield advantage of six lines: 2967, 3008, 3074, 3080, 3085 and 3677 over the non-transgenic Curinga ranged from 10-30% as shown in **Fig. 7**.

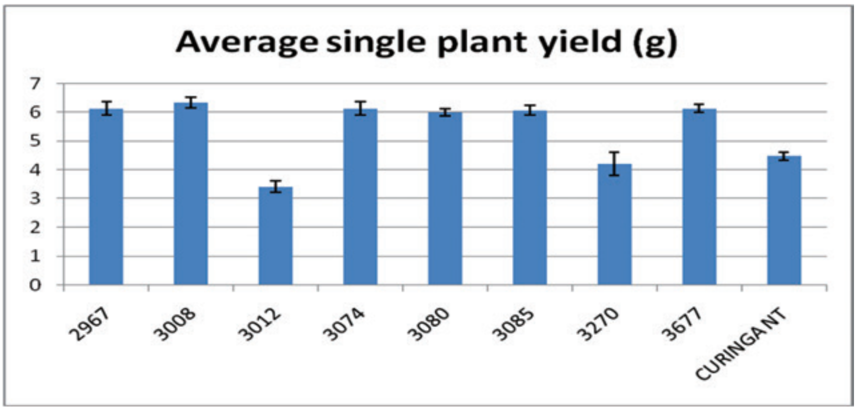

Fig. 7. Variation in single plant yield among the transgenic events of *osnac6::OsNAC6* at the end of drought stress under rainfed conditions.
Curinga NT means Curinga wild type (non-transgenic). Error bar represents ± standard error (SE) (n=15).

Furthermore, the lines 3080 and 3677 had less leaf rolling score and more numbers of productive tillers and better biomass than non- transgenic Curinga (data not shown). It was suggested that the rice OsNAC6 transcription factor is involved multiple molecular mechanisms such as root structural adaptions and nicotianamine biosynthesis for drought tolerance although root specific promoter, *RCc3* was used to ectopically express *OsNAC6* gene in Nipponbare (Lee et al. 2017). Better agronomical performance of the lines used in this study under rainfed conditions should be further confirmed by transgene expression and repeated field trials with bigger plot size.

In 2012 rainfed trial at Santa Rosa, 13 transgenic events of NERICA1 and NERICA4 backgrounds were also evaluated (data not shown). This rainfed experiment revealed that several events from the following genes, namely, *osnac6::OsNAC6* (**Fig. 8**) and *Ubi::AtGolS2* (Selvaraj et al. 2017) performed better than non-transgenic NERICA in terms of single plant yield under stress.

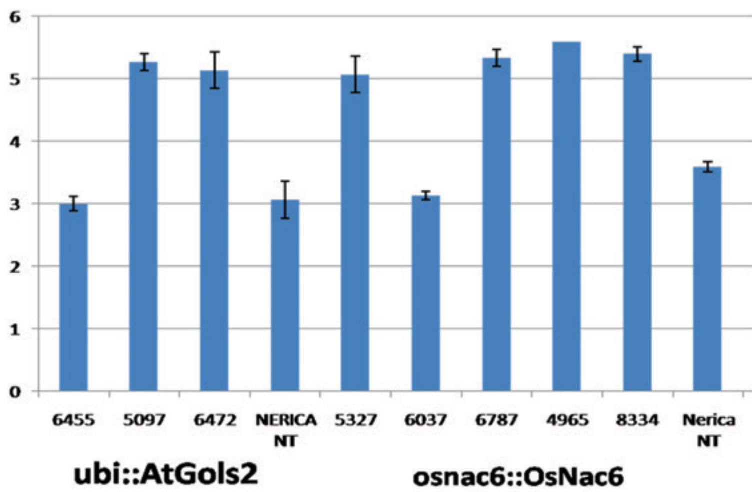

Fig. 8. Variation in grain yield among the different transgenic events of NERICA1 and NERICA4 at the end of drought stress under rainfed trial, 2012-13.
From left to right, genetic backgrounds are NERICA1 for 6455, NERICA 4 for 5097, NERICA1 for 6472, WT and 5327, NERICA 4 for 6037, 6787, 4965, 8334 and WT, respectively. Error bar represents ± standard error (SE) (n=15).

The line 6787, 4965 & 8334 of the construct *osnac6::OsNAC6* had shown more than 35 % yield increase compared to non-transgenic NERICA lines under drought stress. *osnac6::OsNAC6* (**Fig. 7**) and *Ubi::AtGolS2* (Selvaraj et al. 2017) also found to better perform in Curinga background under drought conditions. This finding strongly suggested *OsNAC6* which is to be associated common drought tolerance mechanisms (Lee et al. 2017) can improve agronomical performance under drought conditions regardless of genetic background. Furthermore, OsNAC6 lines with constitutive promoter were not performed well under the studied conditions (data not shown). This indicated suitable combinations of gene and promoter is essential for improved agronomical tolerance in rice.

Conclusion

Through international collaborative research, maximizing institutional strengths on research from basic to application, several genes with different promoter such as *osnac6::OsNAC6* and *Ubi::AtGolS2* were identified, not only improving drought tolerance but also increases grain yield under real field conditions for crop improvement.

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