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ORIGINAL PAPER

Application of T-DNA activation tagging to identify glutamate receptor-like genes that enhance drought tolerance in plants

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Abstract

Key message **A high-quality rice activation tagging population has been developed and screened for drought-tolerant lines using various water stress assays. One drought-tolerant line activated two rice glutamate receptor-like genes. Transgenic overexpression of the rice glutamate receptor-like genes conferred drought tolerance to rice and *Arabidopsis*.**

Abstract Rice (*Oryza sativa*) is a multi-billion dollar crop grown in more than one hundred countries, as well as a useful functional genetic tool for trait discovery. We have developed a population of more than 200,000 activation-tagged rice lines for use in forward genetic screens to identify genes that improve drought tolerance and other traits that improve yield and agronomic productivity. The population has an expected coverage of more than 90 % of rice genes. About 80 % of the lines have a single T-DNA insertion locus and this molecular feature simplifies gene identification. One of the lines identified in our screens, AH01486, exhibits improved drought tolerance. The AH01486 T-DNA locus is located in a region with two glutamate receptor-like genes. Constitutive overexpression of either glutamate receptor-like gene significantly enhances the drought tolerance of rice and *Arabidopsis*, thus revealing a novel function of this important gene family in plant biology.

Keywords Rice · Activation tagging · Forward genetics · Glutamate receptor · Drought tolerance

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Abbreviations

ATL	Activation tagging line
CaMV	Cauliflower mosaic virus
CTAB	Cetyltrimethyl ammonium bromide
CDS	Coding sequence
DsRed	Discosoma red fluorescent protein
GLR	Glutamate receptor-like
RNAi	RNA interference
RT-PCR	Reverse-transcription polymerase chain reaction
T-DNA	Transfer DNA
UTR	Untranslated region

Introduction

Rice (*Oryza sativa*) is a staple food crop for more than half of the world's population. Increasing rice productivity can significantly address the global food security challenge (Zhang 2007). In addition to being an important food crop grown in more than one hundred countries, rice is also a useful genetic tool for gene discovery due to its high throughput genetic transformation, relatively small genome size, complete genome sequence and syntenic relationship with other cereals, and its diverse genetic resources (Zhang et al. 2007; Krishnan et al. 2009). The discovery of rice traits that impart higher yield per acre and allow cultivation under biotic and abiotic stress conditions can positively impact global food production.

Advanced genomic technologies are enabling discovery of genes that can increase crop productivity in a sustainable manner. The use of forward (phenotype to gene) and reverse (gene to phenotype) genetic approaches is an effective way to identify trait genes. Various techniques have been used to facilitate the functional analysis of rice and other plant genes, including generation of loss-of-function mutations from chemical and physical mutageneses (Krishnan et al. 2009; Satoh et al. 2010; Suzuki et al. 2010), from insertions of endogenous or exogenous DNA, such as T-DNA (Hsing et al. 2007) or from transposable elements (Park et al. 2007; Ayliffe and Pryor 2009). Compared with transposons, T-DNA insertions appear to be relatively randomly distributed in a plant's genome (Kolesnik et al. 2004). Thus, loss-of-function populations have been good sources from which to discover rice genes, given a useful assay for the genes of interest (Ryu et al. 2004; Tani et al. 2004; An et al. 2005a, b; Krishnan et al. 2009; Tsuchida-Mayama et al. 2010). Their main limitation is that genes are masked if they act redundantly, if they are compensated for by alternative metabolic or regulatory circuits, or if they are essential for plant growth and development (Weigel et al. 2000; Tani et al. 2004). To overcome these disadvantages, gain-of-function strategies have been employed, which include activation-tagging and full-length cDNA overexpressing gene strategies (Hakata et al. 2010; Tsuchida-Mayama et al. 2010; Higuchi-Takeuchi et al. 2013). Activation tagging is a powerful approach which increases the expression of adjacent genes using transcriptional enhancers randomly inserted in the genome (Weigel et al. 2000; Gou and Li 2012). This method has been successfully used to generate mutant pools in rice (Jeong et al. 2002; An et al. 2005a; Wan et al. 2009), *Arabidopsis* (Pogorelko et al. 2008; Wang et al. 2012), populus (Fladung and Polak 2012; Trupiano et al. 2013), and tomato (Mathews et al. 2003). About 25,000 rice T-DNA insertional mutant lines were generated using the vector pCAS04, which has both promoter-trapping and activation-tagging functions. About 40 % of the lines had single-copy insertions, and the average T-DNA insertion number was 2.3 by Southern blot analysis (Ma et al. 2009). Over 100,000 T-DNA insertion lines were developed in japonica rice (Yi and An 2013), 107,169 insertion sites were identified in this population from 58,943 T-DNA tagged lines (An et al. 2005a; Jeong et al. 2006), and 172,500 flanking sequence tags have been reported in rice (Jung and An 2013). Recently, a transposon-based activation-tagging system has been developed (Guiderdoni and Gantet 2012; Carter et al. 2013; Harb and Pereira 2013). Activation-tagged mutant populations have proven to be a powerful genetic resource for trait discovery and for understanding plant biology (Tani et al. 2004), with populations successfully screened for seed germination (Zhao et al. 2011), plant immune responses (Bao et al. 2013), plant growth and development (Shao et al. 2012; Park et al. 2013), disease resistance (Xia et al. 2004; Grant et al. 2003), and abiotic stress tolerance (Park et al. 2011, 2013).

Abiotic stress is a primary cause of crop losses, causing average yield losses of more than 50 % for major crops (Boyer 1982; Bray et al. 2000). Among the various abiotic stresses, drought is the major factor that limits crop productivity worldwide. Exposure of plants to a water-limiting environment during developmental stages appears to activate cascades of physiological and developmental changes (Xoconostle-Cázares et al. 2010). Although many reviews on molecular mechanisms of abiotic stress responses and genetic regulatory networks of drought stress tolerance have been published (Xiong and Zhu 2001; Wang et al. 2003; Shinozaki et al. 2003; Chaves and Oliveira 2004; Vinocur and Altman 2005; Yamaguchi-Shinozaki and Shinozaki 2005; Valliyodan and Nguyen 2006), it remains a major challenge to fully understand the basic biochemical and molecular mechanisms for drought stress perception, transduction and tolerance. Genetic research has shown

that drought tolerance is a quantitative trait, controlled by many genes. Molecular marker-assisted breeding has led to improved drought tolerance in crops. However, marker accuracy and breeding efficiency remain problematic (Ashraf 2010). Transgenic approaches to engineering drought tolerance in crops have made some progress (Vinocur and Altman 2005; Lawlor 2013).

Glutamate receptors exist in both animal and plant cells (Dean 2002; Davenport 2002). In animals, ionotropic glutamate receptors (iGluRs) represent a class of cation-selective, ligand-gated ion channels mediating excitatory synaptic transmission in the central nervous system (Dean 2002). Plant *GLR* genes were first identified from *Arabidopsis* where they were shown to play a role in light-mediated signal transduction (Lam et al. 1998). In the last decade, various other functions have been attributed to plant *GLR* genes including the regulation of root growth and branching (Liu et al. 2006), the response to aluminum (Sivaguru et al. 2003), ion transport (Kim et al. 2001; Dean 2002; Turano et al. 2002; McAinsh and Pittman 2009), and metabolic and signaling pathways (Lam et al. 1998; Tapken and Hollmann 2008). Suppression of *AtGLR1.1* expression indicated that *AtGLR1.1* functions as a regulator of carbon/nitrogen metabolism, a key step in ABA biosynthesis, and the control of seed germination (Kang and Turano 2003). Overexpression of the *AtGLR3.1* glutamate receptor homolog led to impairment in external Ca^{2+}-induced stomatal closure and imposed Ca^{2+} oscillation-induced long-term stomatal closure without affecting the anion channel activity or the Ca^{2+} oscillation kinetics (Cho et al. 2009). Recent studies demonstrated that *AtGLR3.2*, *AtGLR3.3*, and *AtGLR*3.6 attenuated wound-induced surface potential changes (Mousavi et al. 2013), and the loss of function of certain members of the glutamate receptor-like family of ion-channel proteins affected wound-induced signal generation (Christmann and Grill 2013). A new family of 13 *SlGLR* genes has been identified in tomato and represents the first comprehensive list of *GLRs* in a plant other than *Arabidopsis* (Aouini et al. 2012). Li et al. (2006) identified a rice *GLR* gene which may be involved in cell proliferation and cell survival in the root apical meristem at an early seedling stage. In summary, *GLR*-like genes have multiple functions in plants; no clear role in water stress tolerance has been attributed to rice GLR proteins.

In this study, we report the construction of a population of >200,000 rice activation-tagged lines with advanced molecular features. By screening the population using greenhouse assays, we identified a tagged line exhibiting enhanced drought tolerance. We demonstrated that the drought tolerance phenotype was caused by activation of a *GLR* gene, and that overexpression of either *GLR* gene, *OsGLR1* and *OsGLR2*, significantly improved drought tolerance in both rice and *Arabidopsis*. These results demonstrate that *GLR* genes play important roles in plant abiotic stress biology.

Materials and methods

Plant materials and transformation

Four japonica (*Oryza sativa ssp. japonica*) varieties, Zhonghua 11, Chaoyou 1, Taichong 65 and Nipponbare, were used for the creation of a rice activation-tagging population using *Agrobacterium*-mediated transformation, with the majority of the lines from Zhonghua 11. The transgenic events for trait recapitulation were generated using Zhonghua 11. A 16.3-Kb T-DNA-based binary construct, as shown in Fig. 1, was used to generate the activation-tagging population, which contains four tandem copies of the enhancer of Cauliflower mosaic virus (CaMV) 35S promoter (corresponding to sequences from −341 to −64, as defined by Odell et al. 1985). The binary vector (Construct A) was created by modifying the activation tagging vector of *Arabidopsis*, pHSbarENDs (Aukerman and Sakai 2003), in which pNOS-bar-NOS terminator was replaced by the cassette containing LTP2-DsRed2-PIN terminator–UBI-HygR-NOS terminator. The final construct contained vector sequences (pUC9) and a poly-linker for plasmid rescue, kanamycin resistance for transforming *Agrobacterium*, DsRed for selecting transgenic seeds, ampicillin resistance for plasmid rescue of the T-DNA-flanking sequences, and hygromycin resistance for selecting transgenic plants for screening. Also, the Ds maize transposal element was embedded in the vector to separate 35S enhancer elements from promoters in T-DNA, maximizing the chance of enhancing promoters in the vicinity of T-DNA insertion sites.

Rice transformation essentially followed the *Agrobacterium*-mediated method as described by Lin and Zhang (2005), except for the use of *Agrobacterium tumefaciens* strain AGL0 and that the concentrations of the *Agrobacterium*-harboring plasmid constructs for callus infection were OD600 <0.1. The transgenic seedlings (T0) were rescued and T1 seeds were further advanced to the T2 generation in the field, and then stored in 4 °C.

Arabidopsis thaliana ecotype Columbia was grown in a growth chamber for 1 week under 8-h light/16-h dark and then for 3 weeks at 10-h light/14-h dark at 22 °C day/20 °C night and 70 % relative humidity conditions. *Arabidopsis* was transformed using well-known standard methodology as described by Clough and Bent (1998).

Genetic analysis of T-DNA insertion loci number

A total of 986 random tagging lines were used to estimate the DsRed segregation ratio and T-DNA insertion locus number. Two hundred seeds from each line were used in

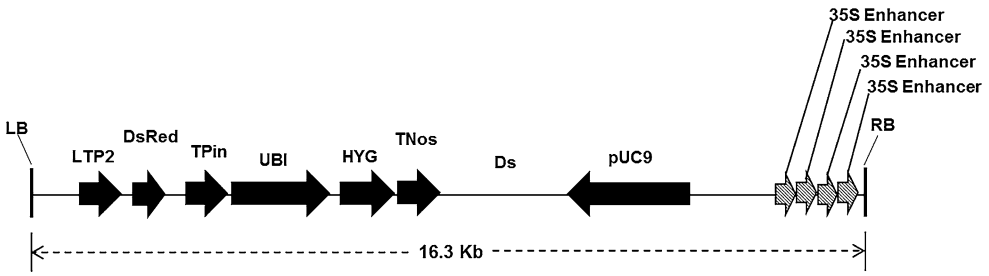


Fig. 1 Functional regions of T-DNA in construct A which was used to generate the rice activation-tagged population. *Arrowheads* indicate direction of transcripts. *RB* and *LB* indicate the right border and left border of T-DNA, respectively. Four tandem copies of the CaMV 35S enhancer were inserted near to the RB. *LTP2* barley lipid transfer protein 2 promoter; *DsRed* Discosoma red fluorescent protein gene; *TPin* Pin2 terminator; *UBI* maize ubiquitin promoter; *HYG* hygromycin resistance gene; *TNos* Nos terminator; *Ds* maize transposal element; *pUC9* pUC9 plasmid skeleton including ampicillin resistance gene

the experiments and the DsRed+ and DsRed– seed numbers were counted under green fluorescent lights. Statistical method $\chi_C^2 = \sum \frac{(|\text{observed}-\text{expected}|-1/2)^2}{\text{expected}}$ was used to test whether the DsRed seed segregation ratio (DsRed+:DsRed–, $r = A/a$) of each line was 3:1 or 15:1. If $\chi_C^2 < \chi_{0.05,1}^2 = 3.8415$, if was $2.2 \leq A/a \leq 4.3$ or $9.1 \leq A/a \leq 36.8$; we accepted that the segregation fitted the ratio of 3:1 or 15:1 at 0.05 levels when $n = A + a = 200$. According to the law of segregation and independent assortment, if the segregation ratio was 3:1 or 15:1, the T-DNA insertion loci number was 1 or 2, respectively.

Greenhouse drought tolerance assay

Transgenic T2 (or T1) seeds of the tagging lines which were red under green fluorescent lights were used for greenhouse drought tolerance screens. Seeds were sterilized with 800 ppm carbendazol for 8 h at 32 °C and washed 3–5 times, soaked in water for 16 h at 32 °C, and then germinated overnight at 35–37 °C in incubator. The germinated seeds were sowed in a tray filled with a mixture of organic soil (FangJie soil from Beijing HuiYeShengDa Center), vermiculite (Beijing QingYuanShiJi Garden Center) and sand (Beijing Shuntun Construction Material Market) (V:V:V = 3:3:2). Twenty uniform seedlings from each line, one positive control (a drought-tolerant variety, Mianhui 501), one negative control (a drought-sensitive variety, Dongbeiyin 2), and one tissue-cultured Zhonghua11line were included in the drought tolerance screens. The seedlings were grown under normal greenhouse conditions (sodium lamp and metal halide lamp at 1:1 ratio to provide long days of 16-h/8-h light/dark, 20–35 °C, and 30–90 % relative humidity) in an IRRI solution (Gregorio et al. 1997). For each line, 20 plants were grown in different positions of the tray. After the seedlings grew to the 3-leaf stage, watering was stopped until the leaves became curved (approximately 9–15 days, depending on the season). Water was added below the trays to enable seedling recovery in 5–7 days, and the plants were scored for their recovery degree. The following scoring system was used: if more than half of a stem was green = 1; if more than two-thirds of a leaf was green = 1; if less than two-thirds but more than one-third of a leaf was green = 0.5; if less than one-third of a leaf was green = 0.2; if no part of a leaf or less than half of a stem was green = 0. The recovery degree was the sum of the scores of the green tissues of a plant, and the data were statistically analyzed using SAS PROC MIXED. The lines, which showed significant differences from the controls ($p < 0.05$) and other mutant plants, were considered to be the primary positive lines. The primary positives were screened again with 32 seedlings. In the third screen, 60 seedlings were included in the experiments under the same condition as the primary screen. The screening data were analyzed using SAS PROC MIXED ($p < 0.05$).

Survival rate (percentage of surviving plants over the total plant number) was also used as a parameter in identifying drought-tolerant tagged lines. Only the lines that passed the third round of screens were considered drought-tolerant lines and were moved into the candidate gene cloning step for recapitulating the trait. The drought tolerance assay for gene recapitulation was carried out similar to the screening of the tagging lines. Nine to 12 events from each construct and 8–16 seedlings from each event at T1 or T2 generations were tested along with the transgenic events from control vector (DP0005 or DP0009) or event null. The results were analyzed by SAS PROC MIXED.

In the *Arabidopsis* drought assays, T1 seeds were sowed in planting soil under normal watering conditions for 4 weeks. The planting soil was watered to saturation before the drought treatment, and the *Arabidopsis* plants grew without watering for about 13 days. When the relative water content of the soil had decreased to about 3–4 %, the *Arabidopsis* plants were re-watered for 4 days, and the plant survival rate then measured. The results were analyzed by SAS PROC MIXED.

T-DNA-flanking sequence and identification of activation-tagged genes

Genomic DNA was isolated from 3-week-old seedling leaf tissues of the tagging lines (AH01486 and other tagging lines) using the CTAB method (Murray and Thompson 1980) to identify the T-DNA flanking sequence by the plasmid rescue method (Behringer and Medford 1992). Ten microgram of genomic DNA was digested overnight with 2 μL each of *Bgl*II/*Hind*III/*Xho*I (NEB, Ipswich, MA) in a 200-μl reaction solution. The digested genomic DNA was self-ligated overnight at 16 °C in a 400-μl reaction solution. The self-ligation sample was transformed into competent *E. coli* DH5α via electroporation. The rescued clones were sequenced using the primer of M13R-46 and the right border-flanking sequences of the T-DNA were determined from the rice genomic annotation in the US National Center for Biotechnology Information of the National Library of Medicine, National Institutes of Health, and the website for the rice genome annotation project at Michigan State University. Based on the T-DNA insertion locus information, the T-DNA insertion site on both right border (RB) and left border (LB) were defined through PCR using a pair of primer complementing the T-DNA and the genomic DNA nearby the T-DNA insertion site, respectively.

GLR gene cloning and vector constructions

To clone the cDNA of rice glutamate receptor-like 1 gene (*OsGLR1*, LOC_Os09g26144.1), two primers were designed based on the sequence information of LOC_Os09g26144.1. The primers were as follows: 56-26144-F: 5′-TCTGCCTGCAGGATGGCTGGTCACA CCCCGAATC-3′ and 56-26144-R: 5′-TAACCTGCAGGT TACTGGCCTCTGCCATTCTCT-3′. The *Sbf*I site was designed in both primers. PCRs were carried out using these primers and pooled cDNA from leaf, stem and root tissues of Zhonghua 11 plants as a template. The PCR product with the expected size of ∼2.8 Kb was purified and then digested by *Sbf*I. The DNA fragment was ligated with *Pst*I-digested vector DP0009 (pCAMBIA1300-35s-NOS-Hyg-deleteAsRED). The resultant construct with the sense orientation was selected as *OsGLR1* overexpression vector DP0025. The *OsGLR1* gene sequences and orientation in DP0025 were confirmed by sequencing.

The rice glutamate receptor-like 2 (*OsGLR2*, LOC_Os09g26160.1) gene was amplified from Zhonghua11 cDNA pool using the following primers which were designed based on the sequences of LOC_Os09g26160.1. The primers were 56-26160-F: 5′-TTTAGGATCCTTGTC CATGGAAGCTG-3′ and 56-26160-R: 5′-ACTCTCGAGT TATTGCCCCCTCTCTGAATG-3′. A *Bam*HI restriction site was added in primer 56-26160-F, and an *Xho*I site in primer 56-26160-R. An expected ∼2.8-Kb PCR fragment was purified and digested by *Bam*HI and *Xho*I. This DNA fragment was ligated with *Bgl*II-*Sal*I-digested DP0005 (pCAMBIA1300-35s-NOS-Hyg) and the *OsGLR2* overexpression vector DP0015 was obtained. The nucleotide sequences and orientation of the *OsGLR2* gene in DP0015 were confirmed by sequencing.

T-DNA was inserted in the second intron of *OsGLR1* in AH01486 mutant. To understand whether the first two 5′ exons of *OsGLR1* in the contributed to the enhanced drought tolerance in AH01486, we cloned the 5′ genomic DNA fragment of *OsGLR1* by PCR, using rice Zhonghua11 genomic DNA as a PCR template. The primers were 56-26144F: 5′-TCTGCCTGCAGGATGGCTGGTCACAC CCCGAATC-3′ and 56-26144R: 5′-CGGCGTTAAACAT TTTGAGATGGAGG-3′. The expected ∼1.7-Kb PCR product was purified and then digested by *Sbf*I. This DNA fragment was ligated with DP0009 which had been digested by *Pst*I +*Nru*I to obtain the overexpression vector DP0011. The nucleotide sequence and orientation of the 5′-part of *OsGLR1* in DP0011 were confirmed by sequencing.

RT-PCR analyses

Total RNA was extracted from about 50 mg leaf, or stem, or root tissues of plants at the 4-leaf stage and cultured in IRRI solution using RNAiso Plus kit (TaKaRa) according to the manufacturer's instructions. The cDNA was prepared by RevertAidTM First Strand cDNA Synthesis Kit (Fermentas) from 500 ng of total RNA. The real-time PCR (RT-PCR) was performed based on the normal procedure using SYBRRPremix Ex TaqTM Kit (TaKaRa) and 7500-Fast real-time PCR equipment (ABI) according to the manual. Gene expression was normalized by the EF1α mRNA levels. The primers used in the RT-PCR analysis for the *OsGLR1* gene and *OsGLR2* gene were as below: *OsGLR1*-3′RT-F: 5′-GACGCCGTACCTGAGGATCTTC-3′; *OsGLR1*-3′RT-R: 5′- CACTTGCGCTCGATGAGGTT C-3′; *OsGLR1*-5′RT-F: 5′-GTCAAGGTCCGGCAAGAA G-3′; *OsGLR1*-5′RT-R: 5′-CTTCCCTCTGTCCATGATG TTC-3′; *OsGLR2*-RT-F: 5′-CTCCTAAAGATATTCCA CG-3′; *OsGLR2*-RT-R: 5′-ACGCTCTCCACATCACTGC-3′.

Sequence alignments

Alignments of the DNA and protein sequences were performed using the Clustal V method (Higgins and Sharp 1989) with the default parameters (GAP PENALTY = 10, GAP LENGTH PENALTY = 10). Default parameters for pairwise alignments and calculation of percent identity of amino acid sequences using the Clustal V method are

KTUPLE = 1, GAP PENALTY = 3, WINDOW = 5 and DIAGONALS SAVED = 5. For nucleic acids, these parameters are KTUPLE = 2, GAP PENALTY = 5, WINDOW = 4 and DIAGONALS SAVED = 4.

Results

The rice activation-tagging population

Several laboratories have developed rice activation-tagging populations and demonstrated the utility of the tagging technology for gene discovery (Jeong et al. 2002; Tani et al. 2004; An et al. 2005a, b; Krishnan et al. 2009; Ma et al. 2009). We have developed a Japonica population of more than 200,000 activation-tagging lines (ATLs) using a modified T-DNA which contains tetramerized CaMV 35S enhancers near the RB (Fig. 1). The number of insertion lines statistically required to tag every rice gene was estimated to be between 180,689 and 460,000 (Hirochika et al. 2004). By RT-PCR analyses of a number of tagged lines, we observed that the tetramerized 35S enhancers as present in the tested activation-tagging construct A could activate rice genes within a region 23 Kb from the RB and 17 Kb

Fig. 2 Various phenotypes of rice activation tagging lines. **a** Normal roots. **b** Long root hairs. **c** Short and more tillers. **d** *Yellow-green* stripe. **e** Semi-sterile panicle with about 15 % of seeding rate. The seeds in **e** are red color due to DsRed expression. These phenotypes were observed from the T2 plants

from the LB, and that, on average, this region (40 Kb) contains 4–5 rice genes. The activated gene expression levels depend upon the genes, their locations, and the tissues (data not shown). We have observed more than 80 types of visible phenotypes associated with root, shoot, leaf, panicle, etc. as indicated in Fig. 2. These phenotypes can be resulted from gene activation or knock-off by the inserted T-DNA or transformation-caused mutations. We are in the process of confirming the phenotypes and studying if the phenotypes are linked with T-DNA insertion. We have demonstrated that a semi-sterile mutant (Fig. 2e) dominantly co-segregated with the T-DNA insertion. These results confirm that the CaMV 35S enhancers in the tested construct configuration can function in rice tissues and activate genes located bi-directionally from the enhancers, within a large genomic region. Based on the present rice genomic data, this rice tagging population is estimated to cover more than 90 % of the rice genes.

The pUC9 plasmid construction in the T-DNA is useful for plasmid rescue of the T-DNA flanking sequences as indicated in the "Materials and methods". The color marker (DsRed gene) is useful for identifying the transgenic tissues and seeds. We selected the transgenic seeds under a green fluorescent light in experiments, and this non-destructive process was significantly simple and cost-effective compared to using antibiotics or molecular techniques to identify transgenic seeds.

Molecular features of the population

Segregation of the color marker can be used to estimate the number of T-DNA loci in the genome. We analyzed DsRed-positive and DsRed-negative seeds of pooled T1 seeds from 986 ATLs which showed that overexpressed *DsRed* could be assigned as a qualitative trait. The statistical analysis indicates that about 74 % (14.81 + 59.13 %) of the tagged lines segregate as a single T-DNA insertion locus ($p < 0.05$), 15 % of the lines have two T-DNA loci, and only 3 % of the lines have more than three T-DNA insertion loci. About 8 % of the lines have either one or two T-DNA insertion loci (Fig. 3a). Plasmid rescue and sequence analyses of 172 tagging lines show that 80 % of the 172 lines harbor only a single T-DNA insertion in the genome, 16 % of the lines have two loci, and less than 4 % of the lines have three or more T-DNA insertion loci (Fig. 3b). Thus, the majority of the tagged lines have only one or two T-DNA insertion loci. This is a significant attribute of population since the presence of single T-DNA insertions simplifies the identification of candidate genes.

To further characterize the molecular features of the population, we analyzed the locations of the T-DNA insertion sites in the rice genome. The 172 tagging lines harbor a total of 213 T-DNA insertion sites which are randomly distributed across the 12 rice chromosomes (data not shown). As shown in Table 1, 59 % of the T-DNAs had been inserted in promoter or terminator regions, and more than 19 % of the T-DNA had been inserted between genes; these are advantageous locations for the gain-of-function mechanism of enhancer-based activation tagging. Notably, about 21 % of the T-DNAs had been inserted into the coding region, inclusive of introns and exons. These types of insertions may also cause loss-of-function and provide a knock-out approach to identifying gene function.

Line AH01486 exhibited significantly enhanced drought tolerance under greenhouse conditions

AH01486 was identified in greenhouse drought tolerance screens. This line showed a rapid and dramatic recovery in

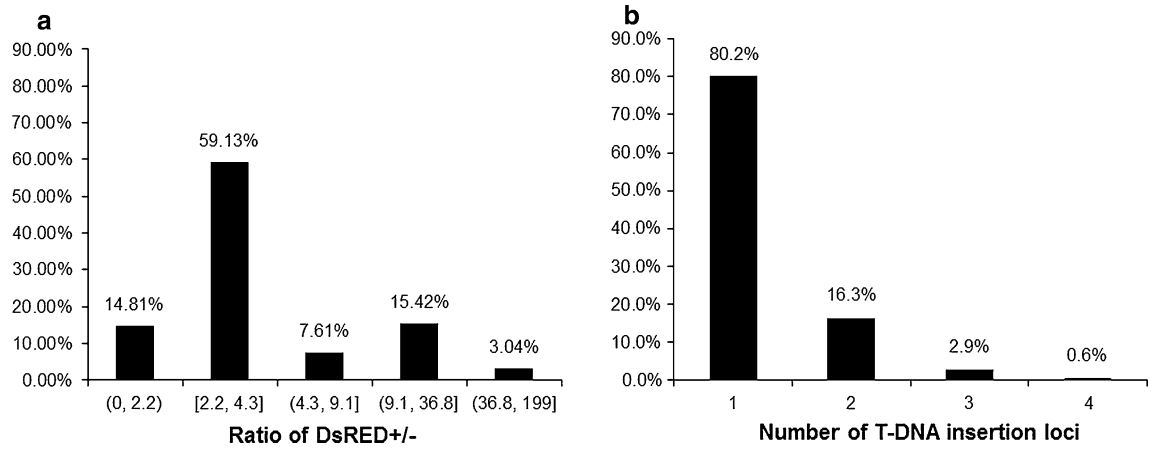


Fig. 3 The number of T-DNA insertion loci in rice activation tagging population. **a** Statistical analysis of DsRed-positive and DsRed-negative seeds from 986 tagging lines. Two hundred seeds from each line were counted and analyzed by Latin-square model. **b** T-DNA insertion numbers of 172 tagging lines analyzed by plasmid rescue and sequence analysis. (0, 2.2), 0 and <2.2; [2.2, 4.3), ≥2.2 and ≤4.3; (4.3, 9.1], >4.3 and ≤9.1; (9.1, 36.8], >9.1 and ≤36.8; (36.8, 199], >36.8 and ≤199

Table 1 T-DNA insertion locations in the rice genome

Location	Number of T-DNA	Percentage (%)
Promoter or terminator	126[a]	59.2
Within a gene	45	21.1
Between genes	42	19.7

Data are from 172 rice activation tagging lines

[a] The total number of T-DNA inserted in 2-Kb and 1-Kb region from the transcription initiation site and terminator site, respectively

10 of 13 plants (77 %) after reintroduction of water following water stress. In contrast, only 33 % of the tissue-cultured Zhonghua 11 control plants survived this treatment. These results indicate that AH01486 plants have significantly enhanced tolerance to water deficit when compared with control plants.

To confirm this observation, we carried out the drought assays using T2 seeds of AH01486. In the first round of screens, 85 % of AH01486 plants survived, while only 35 % of the control survived. The average recovery degree of AH01486 plants was very significantly higher than that of the tissue-cultured Zhonghua11 controls (Table 2, $p < 0.01$). In repeated screens under the same conditions, both the survival rate and the recovery degree of AH01486 plants were consistently and significantly enhanced for drought tolerance compared to the controls (Table 2, $p < 0.05$). These results demonstrate that the AH01486 plants have enhanced drought tolerance under greenhouse conditions at the seedling stage.

T-DNA insertion locus and flanked candidate genes in AH01486

To identify the gene(s) responsible for the drought tolerance phenotype in AH01486, we cloned the T-DNA-flanking fragments using plasmid rescue. The RB- and LB-flanking sequences are shown in Supplemental Fig. 1. Sequence analyses and Blast searches revealed one T-DNA insertion locus in AH01486; its RB was inserted at the 15,760,153 bp and the other border at the 15,760,233 bp of Chromosome 9 (Blasted by MUS 6.1 rice genome database) (Fig. 4). The T-DNA insertion resulted in a 79-bp deletion in rice genomic DNA (based on the rice genome database available in June–July, 2009). The T-DNA in AH01486 has a RB–LB–LB connection. The T-DNA has a 22-bp deletion at the RB and a 65-bp deletion at the 3′ end of the Ubi1 promoter at the LB. The T-DNA was inserted in the second intron of *OsGLR1* (LOC_Os09g26144.1).

Table 2 Drought tolerance assays of AH01486 at T2 generation under greenhouse conditions

Line ID	Screen round	Number of surviving plants	Number of total plants	Survival rate (%)	Average recovery degree	*p* value
Control	1st	7	20	35	0.35	
AH01486		17	20	85	1.09	0.000**
Control	2nd	17	26	65	1.01	
AH01486		29	32	91	1.58	0.020*
Control	3rd	19	60	32	0.61	
AH01486		27	60	45	1.12	0.017*

* and ** indicate the difference between AH01486 and the control at significant ($p < 0.05$) and very significant ($p < 0.01$) level, respectively

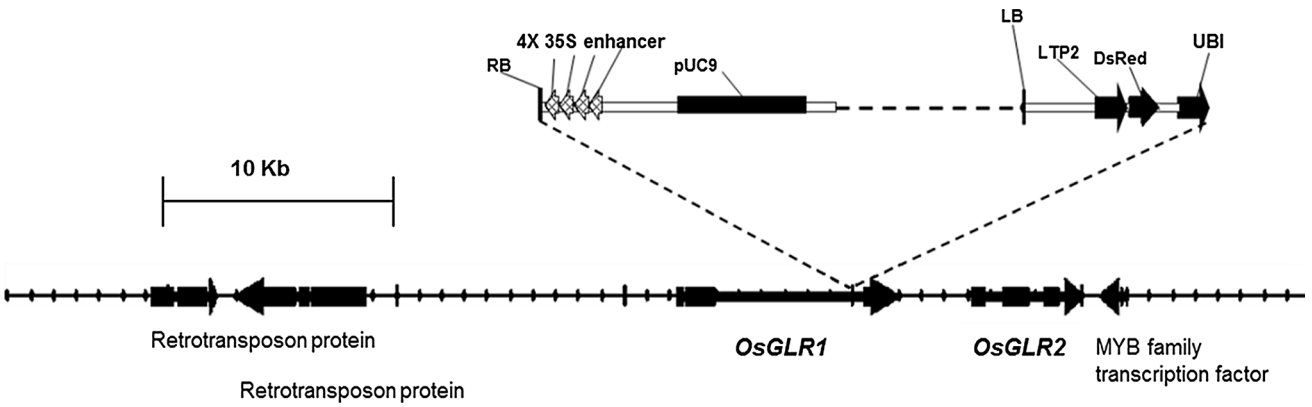
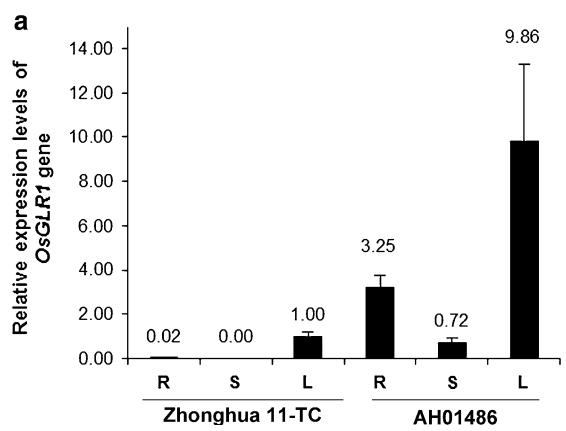


Fig. 4 Map of the insertion position of the T-DNA in AH01486 genome and the nearby genes. The right border (RB) at the 15,760,153 bp and the left border (LB) at the 15,760,233 bp of Chromosome 9, respectively, based on Blast search using MUS 6.1 rice genome database. The T-DNA was inserted into the second intron of glutamate receptor-like 1 gene (*OsGLR1*). *OsGLR2*, rice glutamate receptor-like 2 gene



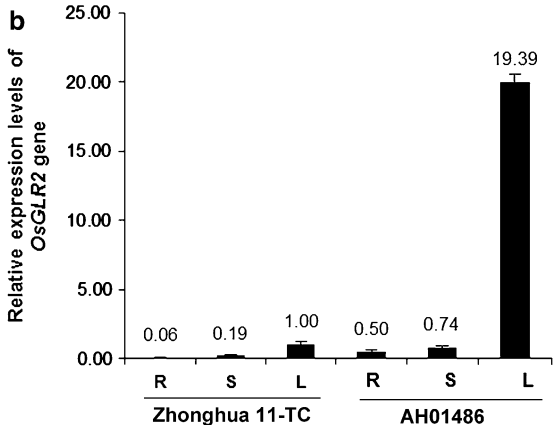


Fig. 5 Activated expression levels of *OsGLR1* (**a**) and *OsGLR2* (**b**) genes in various tissues of AH01486 plants measured by real-time PCR assay. Zhonghua 11-TC, tissue-cultured Zhonghua 11; L, leaves; R, roots; S, stems. *The numbers on the top of the columns* are the fold-increase compared with the corresponding control tissues

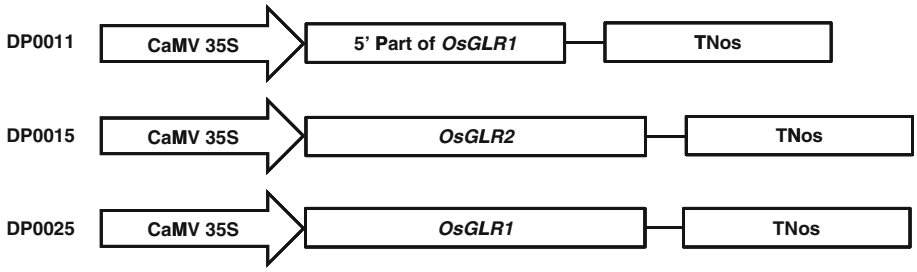


Fig. 6 Schematic diagrams of the overexpression vectors DP0011, DP0015, and DP0025. CaMV 35S, cauliflower mosaic virus 35S promoter; *OsGLR1*, rice glutamate receptor-like 1; *OsGLR2*, rice glutamate receptor-like 2; TNos, Nos terminator

Upstream of the RB, there are two retrotransposon genes. Downstream of the T-DNA LB, there is another glutamate receptor gene *OsGLR2* (LOC_Os09g26160.1) and a MYB family transcription factor gene (Fig. 4).

A functional activation-tagged allele should result in up-regulation of the candidate gene in tissues where it is normally expressed, ectopic expression in tissues that do not normally express that gene, or both. To understand which genes are activated in AH01486, RT-PCR analyses were carried out. The semi-quantitative RT-PCR assays indicated that expression of the MYB transcription factor was unchanged (data not shown). In contrast, as shown in Fig. 5, both the *OsGLR1* (5′ part) and *OsGLR2* genes were significantly activated in root, stem and leaf tissues of AH01486 plants compared to the control. In leaf tissues, *OsGLR1* and *OsGLR2* gene expression levels were increased about 10- and 20-fold, respectively, compared to the control. Since the T-DNA was inserted into the second intron of *OsGLR1*, the activated expression level is from the 5′ part of *OsGLR1* (the first two exons of *OsGLR1*). Based on these results, our phenotype recapitulation efforts focused on these two *GLR* genes.

Both *OsGLR1* and *OsGLR2* genes were cloned using PCR primers based on the information in the rice genome sequence database, and using the rice cDNA pool as the template. Their DNA and protein sequences are listed in Supplemental Fig. 2, 3. The 5′-part of *OsGLR1* was cloned from the genomic DNA and its sequences are shown in Supplemental Fig. 4. There is 37 % identity between *OsGLR1* and *OsGLR2* at the amino acid sequence level across the whole peptides, and 46 % identical amino acid residues in the conserved C-terminal domain. DP0011, DP0015, and DP0025 vectors were constructed to overexpress the 5′ part of *OsGLR1*, full-length of *OsGLR2* and *OsGLR1* under the control of the CaMV 35S promoter (Fig. 6) to understand

Table 3 Enhanced drought tolerance of CaMV 35S Pro:*OsGLR2*-rice plants at T1 generation under greenhouse conditions

Event ID	Number of surviving plants	Number of total plants	Survival rate (%)	Average recovery degree	*p* value
Control	4	16	25	0.25	
DP0015.10	11	16	69	1.22	0.001**
Control	4	16	25	0.25	
DP0015.17	9	16	56	0.98	0.010**
Control	6	16	38	0.39	
DP0015.21	12	16	75	0.93	0.018*

* and ** indicate the difference between transgenic event and the control at significant ($p < 0.05$) and very significant ($p < 0.01$) level, respectively

Table 4 Enhanced drought tolerance of CaMV 35S Pro:*OsGLR2*-rice plants at T2 generation under greenhouse conditions

Event ID	Number of surviving plants	Number of total plants	Survival rate (%)	Average recovery degree	*p* value
Control	4	8	50	0.50	
DP0015.10	7	8	88	1.35	0.017*
Control	0	8	0	0.00	
DP0015.17	4	8	50	0.50	0.019*
Control	4	8	50	0.56	
DP0015.21	8	8	100	1.28	0.020*

* Indicates the difference between transgenic event and the control at significant ($p < 0.05$) level

the contribution of the genes to the improved drought tolerance of AH01486.

Both *OsGLR1* and *OsGLR2* conferred enhanced drought tolerance in rice

The overexpression vectors (DP0011, DP0015, and DP0025; Fig. 6) and the control vectors (DP0005 and DP0009, Supplemental Fig. 5) were transformed into Zhonghua 11. More than 60 independent transgenic events were generated for each vector. The greenhouse drought tolerance assays were carried out at the T1 and/or T2 generations. The transgenic plants were identified by hygromycin selection, and only hygromycin-resistant seedlings were used in the drought assays. Overexpression of either *OsGLR1* or *OsGLR2* genes in rice did not alter plant

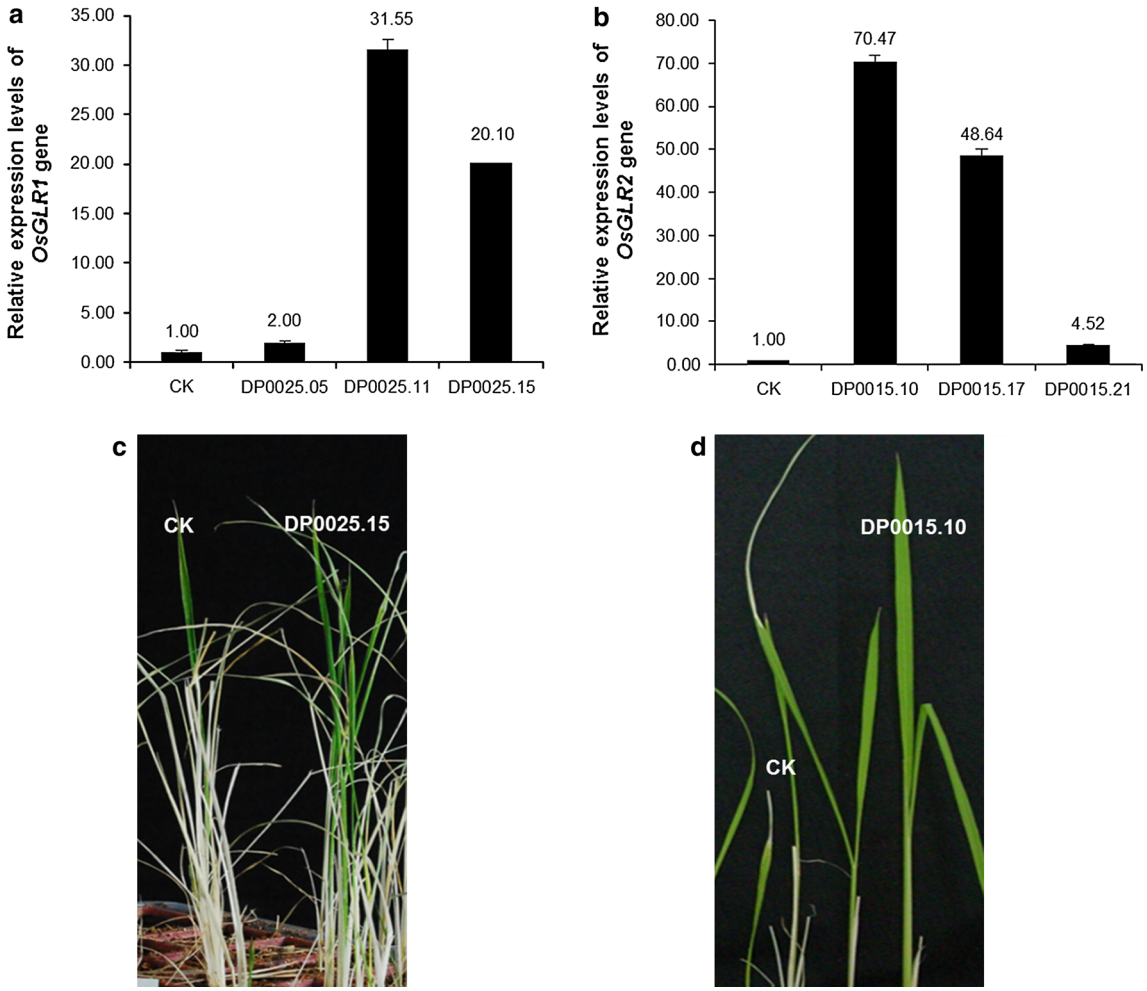


Fig. 7 Transgene expression levels of *OsGLR1* (**a**) and *OsGLR2* (**b**) in the leaves of transgenic events measured by real-time PCR assay. The base level of expression in CK (Control, tissue-cultured Zhonghua 11) event was set at 1.00, and the numbers on the top of the columns are the fold-increase compared with the corresponding control. Phenotypes of DP0025.15 and control plants (CK) (**c**) and DP0015.10 (the two plants on the *right side* of **d**) and its segregated null plants (the two plants on the *left side* of **d**) 5 days after re-watering. The data in **a** and **c** were from T1 plants, and the results in **b** and **d** were from T2 plants

growth and development, and the transgenic plants exhibited same phenotypes as the controls before stop-watering. The greenhouse drought tolerance assays showed that there were no statistical differences among tissue-cultured Zhonghua 11 control, DP0005-transgenic events, DP0009-transgenic events, and event null (data not shown).

As shown in Tables 3 and 4, constitutive (CaMV 35S-driven) overexpression of *OsGLR2* genes resulted in a significant enhancement of drought tolerance under greenhouse conditions. At the T1 stage, overexpression of the *OsGLR2* gene enhanced survival rate and the average recovery degree after water deficit; 67 % of OsGLR2-transgenic rice survived the drought treatment, while only 30 % of the control survived this treatment; the average recovery degree was 1.04 for *OsGLR2*-transgenic rice and 0.3 for the control (Table 3). This indicates that the *OsGLR2*-transgenic plants have enhanced drought tolerance. The transgenic events were further evaluated at the T2 generation. The results in Table 4 and Fig. 7d show that overexpression of *OsGLR2* increased drought tolerance in rice, with the improved tolerance correlated to the transgene expression level (Fig. 7b). These results demonstrate that activated *OsGLR2* expression contributed to the enhanced drought tolerance of AH01486.

As shown in Table 5, the T1 *OsGLR1*-transgenic rice plants show a higher survival rate and average recovery degree compared to the control under moderate drought stress in the greenhouse assays. These results show that overexpression of *OsGLR1* also enhanced the drought tolerance of the *OsGLR1*-transgenic rice plants, and the improved tolerance is correlated to the transgene expression level (Fig. 7a, c). To further understand if the new polypeptide encoded by the truncated *OsGLR1* gene contributed to the enhanced drought tolerance of AH01486, the DP0011 vector was transformed into Zhonghua 11. Overexpression of this truncated *OsGLR1* under CaMV 35S promoter had no effect on drought tolerance in multiple rice transgenic events (Table 6, $p > 0.1$). These results show that the truncated *OsGLR1* did not contribute to the improved drought tolerance of AH01486.

Table 5 Enhanced drought tolerance of CaMV 35S Pro:*OsGLR1*-rice plants at T1 generation under greenhouse conditions

Event ID	Number of surviving plants	Number of total plants	Survival rate (%)	Average recovery degree	*p* value
Control	5	8	63	0.71	
DP0025.05	8	8	100	1.20	0.075
Control	6	8	75	1.13	
DP0025.11	8	8	100	1.83	0.035*
Control	6	8	75	1.13	
DP0025.15	8	8	100	2.30	0.001**

* and ** indicate the difference between transgenic event and the control at significant ($p < 0.05$) and very significant ($p < 0.01$) level, respectively

OsGLR genes enhanced drought tolerance in *Arabidopsis*

Arabidopsis GLR studies demonstrated that plant *GLR* proteins contain "three-plus-one" transmembrane domains (M1–M4) and putative ligand-binding domains (GlnH1 and GlnH2) (Lam et al. 1998), and sequence analysis indicated that the rice *OsGLR1* and *OsGLR2* have the same domains as *Arabidopsis* homologs (data not shown). To understand if rice *GLR* genes could improve drought tolerance in dicot plants, vectors expressing *OsGLR1* (construct DP0025) and *OsGLR2* genes (construct DP0015) were transformed into *Arabidopsis*.

Overexpression of either *OsGLR1* or *OsGLR2* genes in *Arabidopsis* did not alter plant growth and development, and the transgenic plants exhibited same phenotypes as the controls before stop-watering. During the water withdrawal, the wilting levels of control plants (generated from construct DP0009) were more apparent than those of the AtDP0015 and AtDP0025 events, which grew better than the controls after re-watering. On the fourth day after re-watering, 30–70 % of *OsGLR1*- or *OsGLR2*-transgenic plants survived, whereas the corresponding survival rates

Table 6 Drought assays of CaMV 35S Pro:5′ part-*OsGLR1*-rice plants at T1 generation under greenhouse conditions

Event ID	Number of surviving plants	Number of total plants	Survival rate (%)	Average recovery degree	*p* value
Control	13	16	81	1.12	
DP0011.03	11	16	69	0.83	0.219
Control	14	16	88	1.41	
DP0011.08	14	16	88	1.21	0.417
Control	14	16	88	1.41	
DP0011.11	9	14	64	0.94	0.114
Control	14	16	88	1.41	
DP0011.13	12	16	75	1.17	0.377
Control	7	16	44	0.61	
DP0011.15	5	16	31	0.51	0.721
Control	7	16	44	0.61	
DP0011.16	7	14	50	0.71	0.722
Control	7	16	44	0.61	
DP0011.18	4	13	31	0.36	0.335
Control	7	16	44	0.61	
DP0011.21	8	16	50	0.59	0.920
Control	16	16	100	1.35	
DP0011.26	11	16	69	1.12	0.338

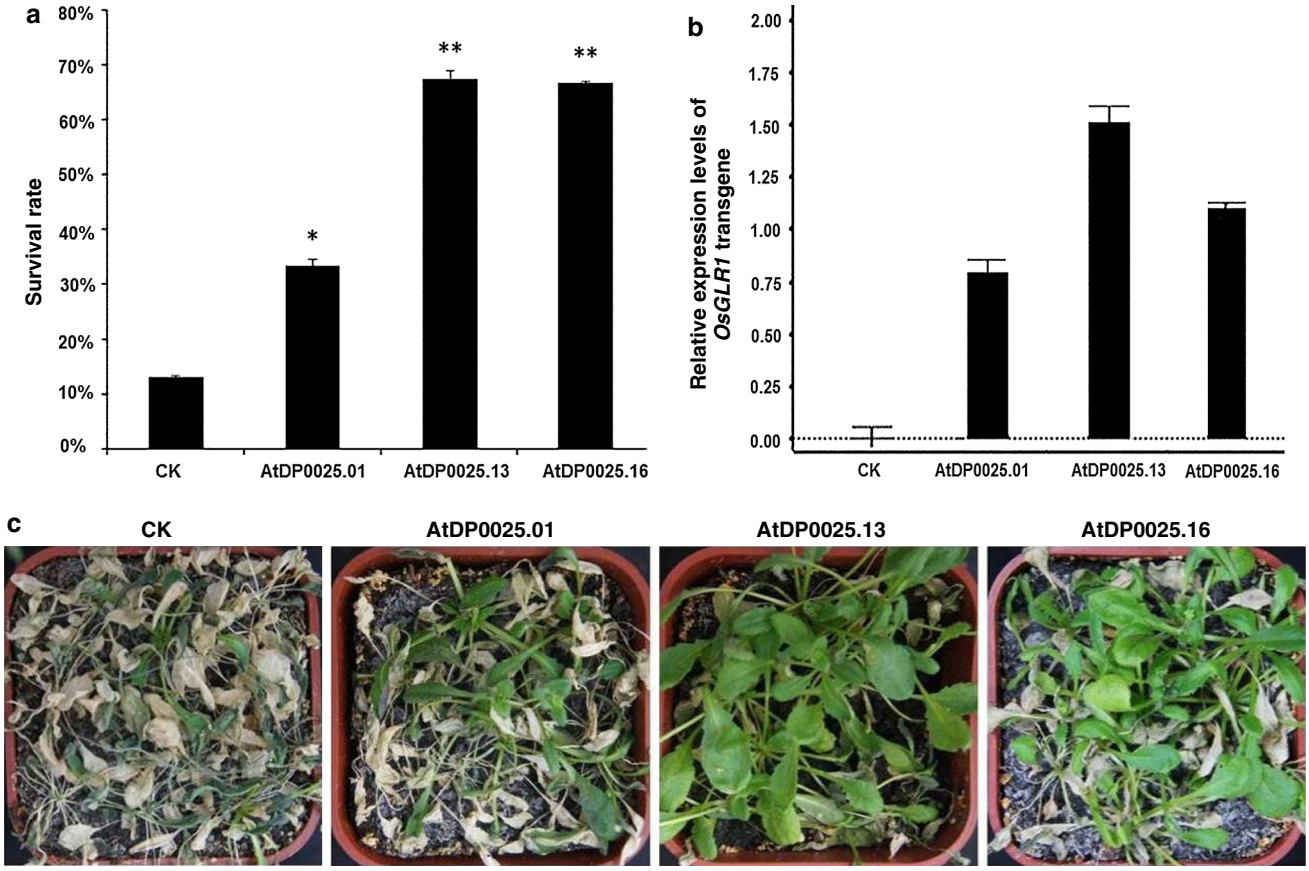


Fig. 8 Overexpression of *OsGLR1* increased drought tolerance in *Arabidopsis*. **a** The survival rate of three DP0025-transgenic events and one control event. **b** Transgene expression levels of *OsGLR1* in the transgenic leaf tissues. **c** Phenotypes of three DP0025-transgenic events and a control event 4 days after re-watering. Data are expressed as mean ± SD at least three independent replications ($n = 3$). *Single asterisk* and *double asterisk* indicate the difference between the transgenic events and the control at significant ($p < 0.05$) and very significant ($p < 0.01$) level, respectively

were <15–20 % for control plants (Figs. 8a, c, 9a, c). The increased survival rates were closely related to the transgene expression levels (Figs. 8b, 9b).

These results are consistent with the results from rice (Tables 3, 4, 5) and demonstrate that overexpression of *OsGLR1* or *OsGLR2* gene can confer enhanced drought tolerance in rice and *Arabidopsis*. In addition, these results further show that a monocot gene (rice *GLR* genes) can function in a dicot plant (*Arabidopsis*), demonstrating the cross-validation of drought tolerance across species.

Discussion

Activation tagging has several advantages over inactivation approaches for uncovering gene functions in rice, *Arabidopsis*, and tomato (Tani et al. 2004). Several rice activation tagging populations have been developed (Jeong et al. 2002; Tani et al. 2004; An et al. 2005a, b; Krishnan et al. 2009). Herein, we report a japonica rice population of 200,000 ATL with several advantages. Theoretically, this size of the population covers >90 % of the rice genome if each tagged line has a single gene activated (Hirochika et al. 2004). However, we found that a single tetramer of the CaMV 35S enhancer activates 4–5 rice genes on average within a 40-Kb region. Therefore, we expect that this population is likely to cover most rice genes. This population has several advantageous features, such as 73–80 % of the tagging lines having a single T-DNA insertion locus, about a quarter of the population having a T-DNA insertion within a gene, the T-DNA being randomly distributed along the 12 rice chromosomes, and a number of lines exhibiting various visible phenotypes.

Activation tagging has been used to study plant growth and development, disease resistance gene discovery, metabolic pathway dissection, and signal transduction (Tani et al. 2004). Herein, we further demonstrated the utility of the activation-tagging technology in agronomic trait gene discovery and stress biology.

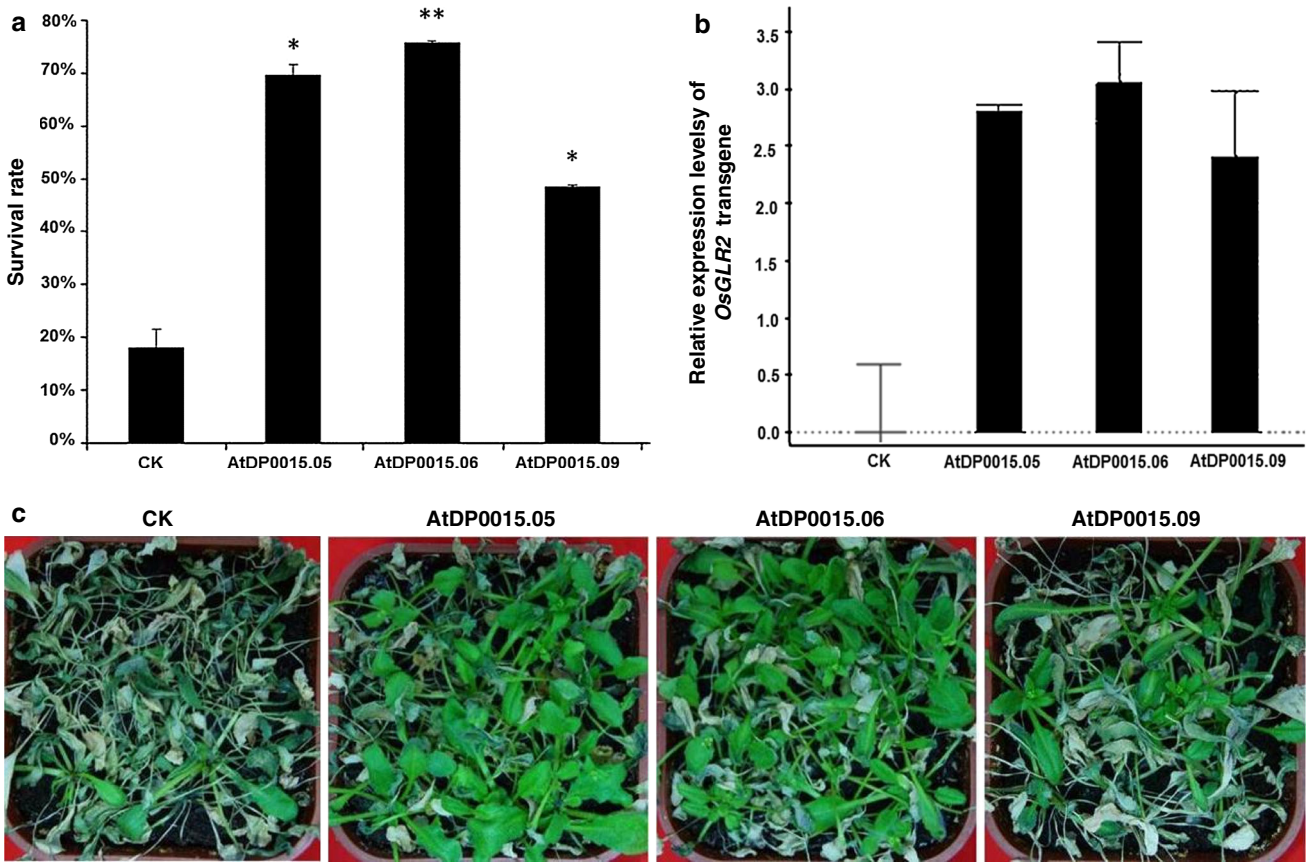


Fig. 9 Overexpression of *OsGLR2* increased drought tolerance in *Arabidopsis*. **a** Survival rate of three DP0015-transgenic events and one control event. **b** Transgene expression levels of *OsGLR2* in the transgenic leaf tissues. **c** Phenotypes of three DP0015-transgenic events and a control event 4 days after re-watering. Data are expressed as mean ± SD at least three independent replications ($n = 3$). *Single asterisk* and *double asterisk* indicate the difference between the transgenic events and the control at significant ($p < 0.05$) and very significant ($p < 0.01$) level, respectively

GLR proteins are found in both animal and plant cells. This gene family comprises diverse signaling pathways and processes, including excitatory synaptic transmission in the central nervous system (Dean 2002), light-signal transduction (Lam et al. 1998), and ion channel function in plants (Tapken and Hollmann 2008). We discovered that two rice *GLR* genes confer significantly enhanced drought tolerance in rice and *Arabidopsis* (Tables 3, 4, 5; Figs. 7, 8, 9). These results demonstrate that *GLR* genes have important roles in response to acute drought stress and in water-use efficiency in plants. Drought tolerance is a complicated trait which requires coordination of multiple responsive pathways (Xiong and Zhu 2001; Shinozaki et al. 2003; Wang et al. 2003; Chaves and Oliveira 2004; Vinocur and Altman 2005; Yamaguchi-Shinozaki and Shinozaki 2005; Valliyodan and Nguyen 2006). Although the mode of action of *GLR* is unknown, the role in drought tolerance may be related to regulation of carbon/nitrogen metabolism and ABA biosynthesis (Kang and Turano 2003) and Ca^{2+}-induced stomatal closure (Cho et al. 2009). The observation that a truncated *OsGLR1* gene could not impact drought tolerance in rice (Table 6) suggests an important role for the C-terminal transmembrane domain (Lam et al. 1998).

Rice transgenic events with high levels of *GLR* expression exhibited healthy and normal architecture (data not shown). The native rice *OsGLR1* and *OsGLR2* genes functioned well in *Arabidopsis* to improve vegetative growth performance under drought stress. These results suggest that ectopic overexpression of rice *GLR* genes can be used to improve agronomic performance of both monocot and dicot crops.

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