RESEARCH ARTICLE

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EXPRESSION OF *SUB1* GENE IN RELATION WITH DIFFERENT SUBMERGENCE TOLERANCE MECHANISMS IN RICE (*Oryza sativa* L.)

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SUMMARY

A cluster of three ethylene response factor (ERF) like genes at the *SUB1* locus has been reported from rice variety FR13A that confers tolerance to submergence for about 14 days and *SUB1* gene-expression was studied. However, *SUB1* gene-expression variation in rice hybrids developed from different tolerant parents is useful to understand distinct submergence tolerance mechanisms. In this investigation, we used semi-quantitative RT–PCR to investigate *SUB1*A and *SUB1*C transcripts in relation with submergence tolerance and stem elongation (under submergence). Two F_1 hybrids were developed, using three distinct parents: susceptible Fulkari, moderately tolerant Kalukanda and tolerant Swarna-*SUB1*. The allele-specific transcripts of two genes in tolerant, susceptible and moderately tolerant varieties indicated that differential expression of both *SUB1*A and *SUB1*C alleles was associated with different level of tolerance. The results demonstrated that the two hybrids had unequal expression of two alleles indicating non-additive interaction of alleles with different level of tolerance. Hybrid-1 (Fulkari/Swarna-SUB1) showed lower negative heterosis for *SUB1*A (-47.8%) and plant survival (-13.9%) and antagonistic interaction of heterozygous combination of two alleles lowered *SUB1*A expression leading to susceptibility. *SUB1* gene-expression in tolerant rice variety Kalukanda and its hybrid (Kalukanda/Swarna-SUB1) showed synergistic action of *SUB1*A and *SUB1*C transcripts with higher *SUB1*A and *SUB1*C, which is a new observation compared with antagonistic relationship (higher *SUB1*A and lower *SUB1*C) reported in tolerant *SUB1* gene. With the possibility of different submergence tolerance mechanisms in Kalukanda, its use in hybridization might lead to improvement of submergence tolerance with balanced levels of both *SUB1*A and *SUB1*C transcripts.

Key words: *SUB1*, alleles, RNA, submergence tolerance, rice (*Oryza sativa* L.)

Key findings: *SUB1A* and *SUB1C* transcripts in rice variety Kalukanda act synergistically (up- regulation of both alleles) indicating different submergence tolerance mechanisms, compared with antagonistic action of *SUB1A* and *SUB1C* transcripts in tolerant FR13A.

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INTRODUCTION

Breeding for improving submergence tolerance in rice cultivars is a major objective for flood prone areas of Asia. Systematic screening of rice germplasm at IRRI has confirmed that FR13A can survive about 14 days of complete submergence. FR13A can thrive in regions affected by flash floods, whereas modern high-yielding varieties cannot survive the flash floods (Mackill *et al*., 2012; Mohanty *et al*., 2000; Xu *et al*., 2006). A cluster of three ethylene response factor (ERF) like genes at the *SUB1* locus has been identified (Xu *et al*., 2006). Two markers (close to *SUB1*) were converted to cleaved amplified polymorphic site (CAPS) markers, through digesting PCR amplified products with respective restriction enzymes and clear tolerant specific *SUB1A1* and *SUB1C1* alleles were found in tolerant accessions. Intolerance was found to be associated with poor submergence induced *SUB1A2* (and *SUB1C2*) or complete absence of *SUB1A* (only *SUB1C* allele). The discovery of *SUB1A* gene facilitated its introgression through Marker Assisted Backcrossing (MABC) to high yielding varieties (Bailey-Serres *et al*., 2010; Collard *et al*., 2013; Mackill *et al*., 2012). Using MABC, *SUB1* gene was transferred into several popular Asian rice varieties (Xu *et al*., 2006; Septiningsih *et al*., 2009, 2013; Thomson *et al*., 2010; Manzanilla *et al*., 2011; Mackill *et al*., 2012; Collard *et al*., 2013). New submergence tolerance rice varieties with *SUB1*A gene might be able to withstand flashfloods affecting vast tracts of paddy.

Flood resistant rice maintains their chlorophyll and underwater photosynthesis (Das *et al*., 2005; Nagai *et al*., 2010; Winkel *et al*., 2013). The physiological response of rice plants to flooding is mainly of two types: (a) restricted elongation ability of leaves and internodes by which the varieties survive under complete submergence, and (b) rapid elongation ability of leaves and internodes by which the varieties avoid the complete submergence, genotypes that elongate faster during flooding are more useful for deep water areas (Chen *et al*., 2011; Luo *et al*., 2011, Sarkar and Bhattacharjee, 2012; Vergara *et al*., 2014). A negative correlation between per cent survival and elongation growth was found, when tolerance is inherited from FR13A (Yamada, 1959; Sasaki *et al.*, 2000a, b; Jackson *et al.*, 1987; Sardana, 1997).

Although FR13A has been successfully used as submergence tolerance source, additional sources are needed. Pyramiding several genes into the same background is the most effective breeding strategy, when multiple genes confer a similar phenotype (Mackill, 2003). Gene based *SUB1* markers were identified by Xu *et al*. (2006) and haplotype-based gene expression analysis of *SUB1* genes has been successfully performed by Xu *et al*. (2006) and Masuduzzaman *et al*. (2017). Different submergence tolerance mechanisms other than *SUB1* gene need to be investigated. In this connection, *SUB1* gene expression analysis in other *SUB1* gene haplotypes and their hybrids are required. *SUB1* gene expression analysis in hybrids is crucial, because breeders are still striving to observe the expression level of *SUB1* gene in hybrid and segregating generations for finding more tolerant genotypes.

RT-PCR (Reverse Transcriptase Polymerase Chain Reaction) analysis is applied to detect differential expression of a specific gene. The number of copies of produced RNA is called the expression level of the gene (Xu, 2005). Gene is "on" when the cell makes mRNA, and "off" when the cell does not make it. Differences in gene expression, termed as expression level polymorphisms (ELPs) (Doerge, 2002) have been studied in many crops and was found to be associated with flowering-time control (Johanson *et al*., 2000; Caicedo *et al*., 2004) and pathogen resistance (Grant *et al.*, 1995; Gassmana *et al.*, 1999) in Arabidopsis. However, for deleterious alleles, synergistic interactions mean that expression of two alleles would be more harmful together than expected from their separate effects. Antagonistic epistasis, therefore implies unexpected robustness to the effects of deleterious alleles (Devisser *et al*., 1996; Peters and Keightley, 2000). Haplotype-based gene expression analysis of *SUB1* genes was studied by many researchers which is relatively reliable than single-locus test. Xu *et al*. (2006) and Fukao *et al*., (2006) described that when *SUB1*A1 gene is very active, it improves the ability of rice to survive under water. They identified the submergence tolerance mechanisms in tolerant,

moderately tolerant and intolerant varieties and reported that *SUB1*A ethylene response factor (ERF) inhibits ethylene production and underwater elongation.

It was confirmed that *SUB1A* gene is the contributor for tolerance (Xu *et al*., 2006; Septiningsih *et al.,* 2009; 2015; Bailey-Serres *et al.,* 2010; Singh *et al.,* 2009, 2015). Expression of *SUB1*A gene also suppresses a nearby ethylene-responsive paralogous gene (*SUB1C*). Masuduzzaman *et al.,* (2010) found A1C1 haplotype as the most tolerant as compared to other haplotypes, indicating the major role of *SUB1A1* for tolerance. Comparison of *SUB1* and non-*SUB1* varieties under submergence indicated that *SUB1A* was the principal determinant in submergence tolerance that inhibited the elongation in *SUB1* lines. In addition, the varieties Madabaru, Kalukanda and Kottamali (A2C2) showed moderate level of tolerance with higher expression of both *SUB1A1* and *SUB1C1* alleles. Submergence tolerant landrace (FR13A) and other *SUB1* haplotypes have been extensively exploited in mechanistic studies; but the study on other *SUB1* gene haplotypes and their hybrids is limited. Efforts are needed to study transcriptional interactions of *SUB1* genes in the F_1s and to investigate strengths of transcriptional interactions of alleles in hybrids for understanding the basis of different tolerance mechanisms.

Effect of nucleotide sequence variation has found on allelic expression patterns, some of which might lead to phenotypic changes (Zhuang and Adams, 2007). Guangming *et al.* (2006) detected unequal expression in rice hybrids and their parents at a leucine-rich repeat receptor kinase gene cluster of different haplotypes and their combinations in hybrids. Analysis of allele-specific expression patterns in the hybrid revealed that high levels of additive (equal to the average of two parents) expression with low levels of non-additive (different from the average of two parents) expression levels (Stupar and Springer, 2006; Swanson-Wagner *et al*., 2006). The expression variation is suggested to play important roles in determining phenotypic diversity in hybrids (Guo *et al*., 2004).

The expression variation is suggested to play important roles in determining phenotypic diversity in hybrids (Guo *et al*., 2004). But, very little is known about *SUB1* allele-specific expression variation in other haplotypes and their hybrid combinations. Current research also focuses on expression variation at *SUB1* locus of hybrids and their parents- in relation with investigating differential mechanism of submergence tolerance. In view of the above mentioned introduction, the present studies were undertaken with the following major objectives: to investigate the relationship between level of *SUB1A* and *SUB1C* transcripts in different haplotypes and their hybrids under submergence stress and also to detect the effect of different patterns of expression of *SUB1A* and *SUB1C* on tolerance level of selected rice hybrids and to understand the basis of different mechanism of submergence tolerance.

MATERIALS AND METHODS

Plant materials and submergence treatment

Two F_1 hybrids (tolerant x intolerant, tolerant x moderately tolerant) and their parents were used in this experiment. For assaying *SUB1A* and *SUB1C* expression, 4 sets of seedlings were raised in separate plastic trays, containing finely ground soil. Except the control tray, other 2 trays with 14 days old seedlings were submerged completely in water tank for 3d and 7d. After submergence of 3d, one tray was taken out of water tank. The shoot tissues were cut into sections (5 to10 mm), and were taken into 2ml tubes, quickly. The samples were frozen immediately in liquid nitrogen and stored at –80°C. Similarly, after 7d of submergence, de-submerged shoots and the corresponding non-submerged controls were also sampled for analysis. All leaves of each variety were harvested at 3 pm on the day of treatment specified and special care was taken not to thaw the samples. The parents and their hybrids (Table 1) were grown in trays and leaf samples were harvested at 7 days after submergence, following same procedures mentioned above.

Table 1. List of parents and F_1s for expression studies.

SL	Varieties	Haplotype	Phanotype
1	F_1-- Fulkari x Swarna-SUB1	-	-
2	F_1-- Kalukanda x Swarna-SUB1	-	-
3.	Parent- Fulkari	A0C2	S
4.	Parent- Kalukanda	A2C2	MT
5.	Parent- Swarna-SUB1	A1C1	T

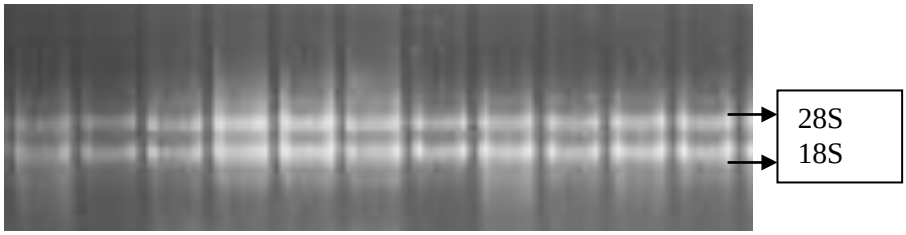

Figure 1. Intact RNA, indicated by clearly visible 18S and 28S rRNA bands.

The data were taken on seedling height, and per cent survival from five randomly selected seedlings, before submergence, after treatment (7 days of submergence) and after the 7th day of recovery.

Measuring elongation and submergence score

Before collecting leaves of submerged plants of each treatment, plant height was measured. To observe the response of increased elongation growth on gene expression, elongation was calculated, as compared with initial plant height (non-submerged control) of each variety. The trays were kept in screen house for recovery of seedlings. The data were taken on seedling height, and per cent survival from five randomly selected seedlings, before submergence, after treatment (7 days of submergence) and after the 7th day of recovery.

Total RNA extraction

Total RNA from shoot tissue samples was extracted following TRI Reagent (Sigma Aldrich, USA) based protocol. Frozen tissue sample (~ 80-100 mg) was taken in a prechilled mortar. It was then powdered by grinding in mortar and frequently or periodically adding liquid nitrogen in the mortar to prevent thawing. Finally, dried RNA pellets were re-suspended in100µl Diethyl Pyrocarbonate (DEPC) treated double distilled water.

Quality check of RNA

RNA samples and amplification reactions were assembled on ice and precautions were taken to save the samples from contamination and degradation by RNase. RNase-free tips and plastic tubes and solutions (treated with DEPC to inactivate RNase) were used. RNA integrity was assessed by comparing the relative intensities of the 28S and 18S rRNA bands (Figure 1) in 1.5% agarose gel electrophoresis, stained with SYBR safe (Thermofisher).

Total RNA concentration and purity (260/280 ratio) were also measured in duplicate by the NanoDrop spectrophotometer (NanoDrop Technologies). Only the RNA samples having a 260/280 ratio between 1.8 and 2.0 were used. Subsequently, the samples were diluted with nuclease-free water to a concentration of 100 ng/µl. The diluted 10 µl of each RNA sample was treated with the RNase-free DNase for 30 min at 37°C (Promega, Madison, WI) to remove traces of contaminating DNA, followed by treating with RNase inhibitor for 10 minutes at 65°C in 0.5 ml tubes in a thermal cycler,

Table 2. Nucleotide sequence of gene specific primers and thermo cycling conditions for RT-PCR.

Primers	Sequence of primers	Annealing Temp (°C)	No of cycles	PCR product size (bp)
SUB1A	F: 5´-GAT GTG TGG AGG AGA AGT GA-3´ R: 5´-TGT TTT GGT GGA TCG ATG GG-3	54	33	203
SUB1C	F: 5´-AAC GCC AAG ACC AAC TTC C-3´ R: 5´-AGG AGG CTG TCC ATC AGG T-3´	53	34	173
Actin-1	F: 5´-ACA GGT ATT GTG TTG GAC TC-3´ R: 5´-GCT TAG CAT TCT TGG GTC C-3´	53	35	118

according to the manufacturer's instructions (Promega). Then concentration of purified RNA was measured again in duplicate by the NanoDrop. Finally, samples were diluted to a conc. of 33.3 ng/µl and the RNA stock solutions were stored at -20 °C.

Semi-quantitative RT-PCR for cDNA synthesis

For first stand cDNA synthesis in a single tube, semi- quantitative RT-PCR was performed, using one step RT-PCR kit (Invitrogen). Totally 100 ng (3µl) of high-quality total RNA was used as a template. The RT reactions were performed in a 25 µl reaction mixture consisting of 22 µl of the RT-PCR reagent mixture and 3µl of total RNA (100ng). For one reaction, the RT-PCR reagent mixture contained: RNase-free water 6.75µl, 12.5 µl of 1X one-step RT-PCR buffer (Invitrogen, Carlsbad CA), 1 µl gene specific forward and reverse gene specific primers mixture, DMSO 1.25 µl and 0.5 µl of one-step RT-PCR/ Platinum Taq mix (Invitrogen, Carlsbad CA), and over layered with 1 drop of nuclease-free mineral oil (Sigma), according to the manufacturer's instructions. After reaction assembly, the tables were transferred to a thermal cycler (G-Strom) (pre-heated to the desired cDNA synthesis temperature of 50°C) and immediately the RT-PCR amplification program was started with 1 cycle of reverse transcription at 50°C for 30 min and then PCR condition consists of: initial denaturation for 5 min at 94ºC, followed by 30 to 33 cycles of 95ºC for 15s, annealing at 53-55ºC for 30s and extension at 72ºC for 1 minute, and a final extension step at 72ºC for 8 min. In thermal cycler, cycling condition was optimized for each primer pairs to ensure amplification products did not reach saturation.

To control contamination from previous sample, a control reaction without RNA template was set up. For more reliable results, three replicates were performed for each sample. The mRNA levels were determined for *SUB1A* and *SUB1C* and for one housekeeping gene: actin-1. The transcript levels were compared across multiple samples, based on band intensity of amplification products relative to internal control (actin1) those were expressed at a relative constant level among all samples.

Gel electrophoresis of PCR products

To quantify the gene expression of *SUB1A* and *SUB1C* and *Actin*-1, the RNA samples were run on 1.5 % agarose gel electrophoresis. PCR products were run at constant voltage 80 V and the gel was stained in CYBR safe for 30 minutes. The amplified products were visualized under UV light and documented by gel documentation system.

Heterosis for the traits related to tolerance

We treated seedlings of both hybrids (involving contrasting traits for elongation, survival and *SUB1A* and *SUB1C* transcripts) under 7d of submergence and assayed the effect of allele-specific transcript accumulation on elongation growth. Same line, Swarma-*SUB1* was used as donor of tolerant specific *SUB1A* and *SUB1C* alleles in two crosses (Table 2). The interactions of different alleles are hypothesized to be the basis of differential expression in hybrids. The average of two parents was used to estimate mid-parent heterosis. To determine the

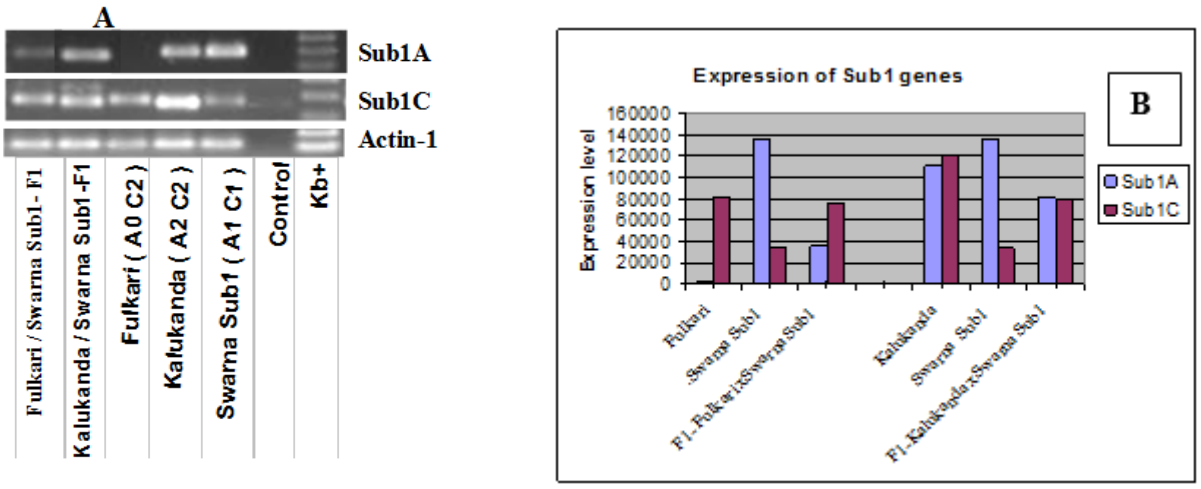

Figure 2. RT-PCR detection of *SUB1A, SUB1C* and Actin-1 transcription in hybrids and their parents at 7d of submergence. *A*: Representative RT-PCR gel for 2 hybrids with respective parents B: Densitometry analysis of mRNA expression for *SUB1A* and *SUB1C.*

significance of heterosis, t-test was utilized. We considered epistasis as any deviation from additivity of the genotype values, looking at the interaction of alleles at the heterozygous state.

Data Analysis

The specific band intensity for *SUB1A* and *SUB1C* with respect to a housekeeping gene (actin-1) was compared from semi- quantitative RT-PCR data at two-time points (3 days and 7 days of submergence. Relative transcript abundance was calculated using the comparative cycle threshold method (Livak and Schmittgen, 2001). The intensity of each band of *SUB1A*, and *SUB1C* was measured, using an IS-1000 Digital Imaging System (Alpha Innotech) and quantified using Flurochem version 2.0 software (AlphaEaseF Canalysis software). The normalized intensity values were detected in comparison with the lowest negative control '0'. Bar graphs represented the relative band intensity of *SUB1A* and *SUB1C* at 7d of submergence. Expression values and elongation means of genotypes were computed.

Gene expression was treated as quantitative trait (Gibson and Weir, 2005) to find out interactions between alleles (Phillips, 1998; Brem *et al*., 2005). If expression of two interacting alleles increase in a positive skewness (higher value), synergistic epitasis occur. If one allele increases in the positive skewness and other one decreases, then antagonistic epistasis occurs. Finally, we investigate the differential mechanism of submergence tolerance in relation with average survival and elongation of different varieties due to differential expression of *SUB1A* and *SUB1C* in hybrids and their parents.

RESULTS AND DISCUSSION

Allelic expression variations in hybrids and their parents

To distinguish between these possibilities, the expression of *SUB1A* and *SUB1C* alleles were compared in two F_1 hybrids involving three selected rice varieties: susceptible Fulkari (A0C2 haplotype), moderately tolerant Kalukanda (A2C2 haplotype) and tolerant Swarna-SUB1 (A1C1 haplotype). The parents and their hybrids, Fulkari x Swarna-SUB1 and Kalukanda x Swarna-SUB1) were submerged for 7 days to investigate gene expression variation in hybrids. Figure 2 showed the *SUB1A* and *SUB1C* transcript of hybrids along with their parents. In tolerant Swarna-SUB1, the expression of *SUB1A* was highest; while

Table 3. Percent heterosis (mid parent) for different traits and allele-specific transcripts under 7d submergence stress.

Expression/ Phenotype	Fulkari	Swarna-SUB1	Hybrid 1	MpH%	Kalukanda	Swarna-SUB1	Hybrid-2	MpH%
SUB1A(E)	2	136	36	-47.8*	111	136	81	0.53
SUB1C(E)	60	38	64	30.6*	120	38	80	1.3
Elongation %	156	42	137	38.4**	70	42	59	5.4
Survival %	5	90	41	-13.9*	67	90	73	-8.9*
Remarks	S	T	S		MT	T	T	

MpH% = Mid parent heterosis %, E = Expression value; *, ** and *** indicated significant at 5%, 1% and 0.1% levels of probability, respectively.

expression level of *SUB1C* was very low. In susceptible Fulkari, the expression of *SUB1C* was highest and *SUB1A* was not expressed. Thus, the allele-specific transcripts of two genes indicated that parental alleles had higher allelic expression differences.

In hybrid-1 (Fulkari/Swarna-*SUB1*), *SUB1A0/ SUB1A* (involving null and normal allele) and *SUB1C1/SUB1C2* (length variation and position of SNP) heterozygous allelic combination resulted in novel interactions those may be the sources of expression difference of *SUB1A* and *SUB1C*. *SUB1A* and *SUB1C* expression level in hybrid-1 was not intermediate between two parents, but shifted towards (higher *SUB1C* and lower *SUB1A*), that of the homozygous parent, Fulkari. As the effect at two loci was not additive; it indicated the involvement of interaction for variation of transcripts.

Expression analysis also indicated that both *SUB1A* and *SUB1C* alleles were expressed in the hybrid-2 (Kalukanda/Swarna-SUB1). The expression level skewed to homozygous parent, Kalukanda. Kalukanda had single-nucleotide polymorphism at *SUB1A* locus compared with Swarna-SUB1. In hybrid-2, *SUB1A1/SUB1A2* (involving SNP variation) and *SUB1C1/SUB1C2* (length variation and position of SNP) heterozygous allelic combination might play a vital role in regulation of *SUB1A* and *SUB1C*. *SUB1* gene-expression studies in Kalukanda and its hybrid (Kalukanda/Swarna-*SUB1*) showed that *SUB1A* and *SUB1C* transcripts act synergistically (higher *SUB1A* and *SUB1C*) and Kalukanda has different submergence tolerance mechanisms compared with antagonistic relation (higher *SUB1*A and lower *SUB1*C) in tolerant *SUB1* gene of FR13A (Xu, *et al.,* 2006). The presence of novel submergence tolerance gene in Kalukanda and Madabaru was first discovered by Masuduzzaman *et al.* (2010) and after that investigation - Septiningsih *et al.* (2013) searched for novel quantitative trait loci (QTLs) from a cross between IR72 and Madabaru. They identified four QTLs on chromosomes 1, 2, 9, and 12 and suggested that an alternative pathway may be present in those varieties different from *SUB1*A gene. These findings also proved the novelty of Kalukanda and Madabaru with distinct submergence tolerance mechanism than the mechanism of FR13A. Further, *SUB1A* and *SUB1C* expression values in hybrid-2 were not equal to the average of both parents, indicating involvement of interaction for transcript variation as earlier reported in maize (Song and Messing, 2003; Guo *et al.,* 2004), wheat and rice hybrids (Bao *et al*., 2005; Wang and Sadee, 2006) for significant allelic expression difference in hybrids. The genetic control of allelic expression variation has been documented in yeast, mice and maize hybrids (Brem *et al.,* 2002; Schadt *et al.,* 2003; Yvert *et al.,* 2003). Heterosis did not simply resulted from overall genetic diversity within a hybrid, but is likely a reflection of diversity at specific genes those contributed to a particular trait (Stuber *et al*., 1992; Xiao *et al*., 1995; Li *et al*., 2001; Luo *et al*., 2001).

Heterosis in expression and tolerance level of hybrids

Allele-specific transcript accumulation of *SUB1*A and *SUB1*C genes were assayed in two hybrids, involving contrasting traits for elongation growth. Same line, Swarna-*SUB1* was used as donor of tolerant specific *SUB1A1* and *SUB1C1* alleles and marked differences existed between the parental mean and F_1 generations in the two crosses for the traits studied, as shown in Table 3. Hybrid-1 showed low negative heterosis for *SUB1A* (-47.8%) and survival (-13.9%). In fact, *SUB1A* transcript and survival % was much lower than the mid parent (MP) values and the concept of epistasis model fitted in the hybrid-1 for explanation of variation for these traits. Hybrid-1 showed positive heterosis for *SUB1*C (30.6%) and elongation (38.4%), which might be due to the detrimental effect of lower *SUB1*A expression. Hybrid-2 showed slightly higher positive heterosis for *SUB1A* (0.53%) and survival per cent (5.4%), but *SUB1A* transcript and survival per cent were different from MP value and the concept of epistasis model also best explained the variation in the hybrid-2. Hybrid-2 also had slightly higher positive heterosis for *SUB1C* (1.3%) and elongation (5.4 %). In fact, almost equal portion of *SUB1A* and *SUB1C* did not exert much detrimental effect of survival in hybrid-2. Thus, selection of hybrid-2 might lead to improvement of tolerance with balanced level of *SUB1A* and *SUB1C* transcripts. Similar results were found by Xu *et al.,* (2006) and *SUB1* introgressed cultivars have also shown higher tolerance and same mechanisms of tolerance in rice (Iftekharuddaula *et al*., 2011; Mackill *et al*., 2012; Septiningsih *et al*., 2009, 2013, 2015; Singh *et al*., 2009).

Effect of differential expression of *SUB1A* and *SUB1C* on elongation

The allelic variation in gene expression in two F_1 hybrids was determined; involving slow, medium and strong elongating parents: 1) hybrid-1, Fulkari/Swarna-SUB1 (strong elongating x slow-elongating) and hybrid-2, Kalukanda/Swarna-SUB1 (medium elongating X slow-elongating). We analyzed the interactions of *SUB1* locus genes and their effect on elongation and fitness (survival**)** (Figures 3 and 4). According to reported studies, interactions between alleles at *SUB1* locus played significant role on expression of elongation and tolerance level of rice. Several studies have also focused importance of *SUB1* gene for reduced elongation under submergence conditions (Baxter *et al*., 2014; Fukao *et al*., 2011; Liu *et al*., 2015; Yang and Hong, 2015). Thus, deployment of tolerant varieties with *SUB1A* gene has been found highly effective (Singh *et al*., 2009; 2013; Iftekharuddaula *et al*., 2011; Dar *et al*., 2013).

Epistasis interaction is defined as a deviation from additive gene effects (Lynch and Walsh 1998). As expression level of *SUB1A* and *SUB1C* genes were deviated from additive effect, epistasis model can best explain the basis of heterosis rather than dominance and over dominance hypothesis (Hull, 1945) in the two hybrids. Interactions in heterozygous states were best explained by either antagonistic or synergistic effect. In hybrid-1, *SUB1A* and *SUB1C* interacted antagonistically reducing the amount of *SUB1A* transcripts (but slightly higher than the lower parent) and up-regulated the *SUB1C.* Finally, expression of strong elongation indicated susceptible phenotype in hybrid-1 (Figure 3). Both hybrid-1 and Fulkari are susceptible, but hybrid-1 had slightly higher survival percent (41%) than Fulkari (5%). The deviation in fitness might be due to a change in strength of interactions raised from differential expression of *SUB1A and SUB1C* alleles in hybrid-1 compared with the parent, Fulkari.

In hybrid-2, *SUB1A* and *SUB1C* alleles interacted in a synergistic manner–thus increasing level of both *SUB1A* and *SUB1C* transcripts. Finally, moderate level of elongation contributed to medium level of tolerance, as like in Kalukanda. Hybrid-2 and Kalukanda are moderately tolerant for their range of survival, but hybrid-2 had slightly higher survival (73%) than Kalukanda (67%). The deviation in fitness might be due to a change in strength of interactions raised from differences in expression of *SUB1A* and *SUB1C* alleles in the hybrid-2 compared with the parent, Kalukanda. Analyzing the involvements of transcript variation in differentiating xylem of *Eucalyptus* wood-forming tissues, Kirst *et al.* (2005) found interactions of the interacting alleles for different traits in an F_1 hybrid. A number of interactions

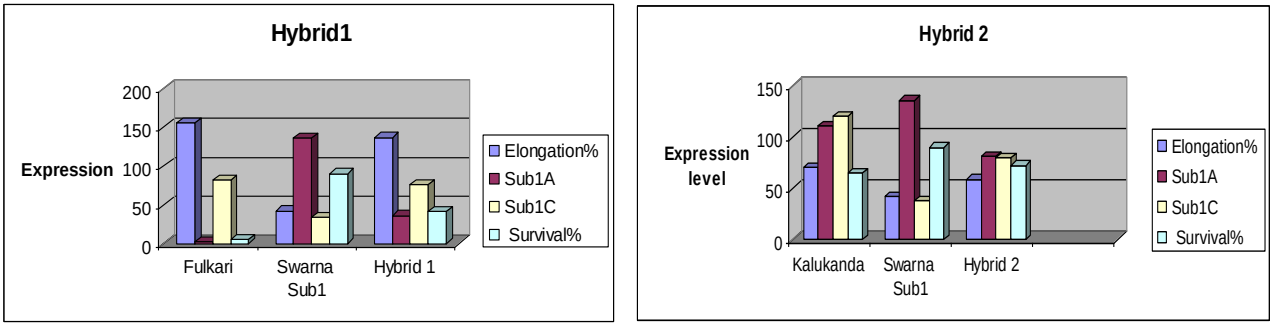

Figure 3. Comparing expression of *SUB1A* and *SUB1C*in relation with elongation and survival percent in hybrid and their parents. Short bars represent smaller effect and long or medium long bars represent greater effects. A. In hybrid 1, antagonistic interaction of *SUB1A* and *SUB1C* alleles favored suppression of *SUB1A* and expression of *SUB1C* as well expression of higher elongation. The presence of higher elongation caused lower survival percent. B. In hybrid 2, synergistic interaction of *SUB1A* and *SUB1C* alleles favored suppression of both the alleles and medium elongation. The presence of strong elongation caused lower survival percent.

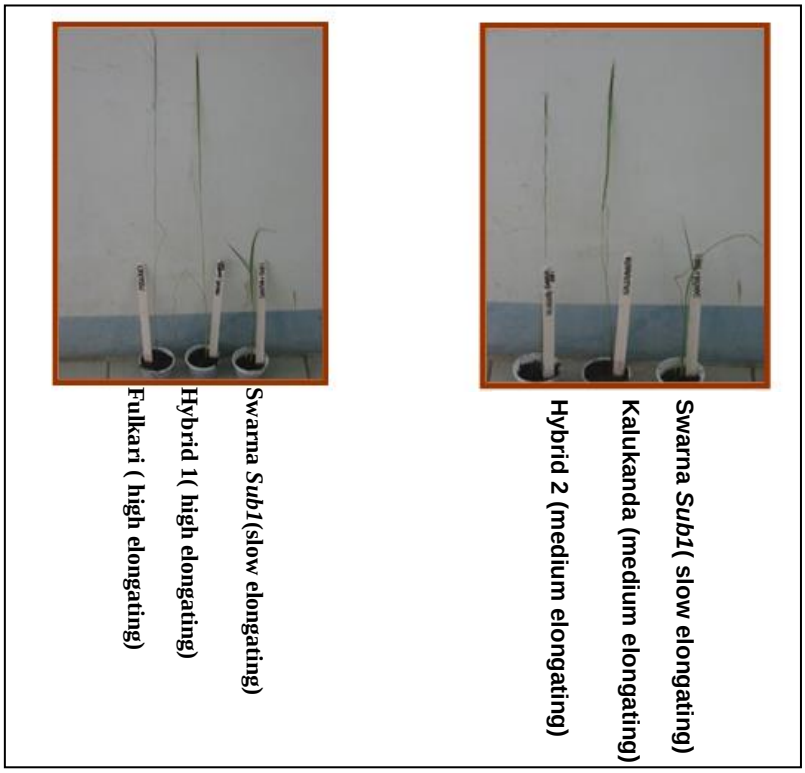

Figure 4. Phenotypes of two hybrids compared with their parents.

among the several genes have been found in Arabidopsis accessions and F1 hybrids using cDNA array (Devisser et al., 1996). These results suggested that major allele for elongation of Kottamali should be a particularly good candidate for the source of novel genes for submergence tolerance. These results are distinct from several studies in rice for submergence

tolerance (Xu *et al.*, 2006; Singh *et al*., 2009; among the several genes have been found in Arabidopsis accessions and F_1 hybrids using cDNA array (Devisser *et al*., 1996). These results suggested that major allele for elongation of Kalukanda should be a particularly good (Jantaboon *et al.*, 2011; Bailey-Serres *et al*., 2012; Dar *et al*., 2013).

In the two hybrids, the expression pattern of *SUB1A* and *SUB1C* affected elongation growth differentially (Table 3). In fact, moderate level of elongation (59%) of hybrid-2 was found better for higher survival, compared with high level of elongation in hybrid-1(137%). A novel mechanism favored the expression of both the *SUB1A* and *SUB1C* alleles that caused limited leaf chlorosis and moderate level of elongation in hybrid-2, as like Kalukanda. These results further indicated the presence of novel beneficial allele in Kalukanda that resulted in beneficial contribution to *SUB1A* and *SUB1C* expression and higher survival. Troyer (2006) pointed out the importance of adapted or beneficial alleles in heterosis of corn. Selection of hybrid-2 (Kalukanda/Swarna *SUB1*) might lead to improvement of tolerance having balanced level of *SUB1A* and *SUB1C* transcripts.

CONCLUSION

In this investigation, using semi-quantitative RT–PCR - *SUB1*A and *SUB1*C transcripts were interpreted in respect with submergence tolerance and stem elongation (under submergence). Two F_1 hybrids were developed, using 3 distinct parents: susceptible Fulkari, moderately tolerant Kalukanda and tolerant Swarna-*SUB1*. *SUB1* gene-expression in hybrids compared with parents (different *SUB1* gene haplotypes) showed that two hybrids had unequal expression of two alleles and had different level of tolerance, compared with respective parents. The shift in expression patterns in *SUB1A* and *SUB1C* of hybrids was due to non-additive interaction of alleles that also affected the elongation and survival of different hybrids differentially. Hybrid-1 (Fulkari/Swarna-*SUB1*) showed lower negative heterosis for *SUB1A* (-47.8%) and survival (-13.9%) heterozygous combination of two alleles interacted antagonistically (lower *SUB1A*) that showed toward susceptible phenotype. *SUB1* gene-expression in tolerant rice variety Kalukanda and its hybrid (Kalukanda/Swarna-*SUB1*) showed that *SUB1A* and *SUB1C* transcripts act as synergistically (higher *SUB1A* and *SUB1C*), which is a new innovation compared with antagonistic relationship (higher SUB1A and lower *SUB1C*) in tolerant *SUB1* gene. Different submergence tolerance mechanisms might be present in Kalukanda. Use of Kalukanda in hybridization and selection of its hybrid might lead to improvement of submergence tolerance having balanced level of both *SUB1A* and *SUB1C* transcripts.

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