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Identification of Candidate Gene *SalT* and Designing Markers Involving in Salt Tolerance of Vietnamese Rice Landraces

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Abstract. Rice (*Oryza sativa* L.) is a principle crop as the main economic importance in Vietnam, providing daily food for over 90 million people in this country. However, a large rice growing areas and rice production are being seriously affected by salinity intrusion, the threats of devastation from climate change. The need to develop salinity tolerance rice varieties to cope with adverse climate change is very imperative. In this study, based on the genome sequence databases of 36 Vietnamese rice landraces, we have identified nine Vietnamese rice landraces carrying nine *SalT* candidate genes with the sequence similarity to *O. sativa SalT* (the published GenBank: Z25811.1) which have shown salinity tolerance are included: Te Nuong, Khau mac buoc, Chan thom, Khau giang, Tan ngan, Nang thom cho dao, OM5629, Hom rau and Thom Lai landraces). Amongst them, four rice landraces, Nang thom cho dao, OM5629, Hom rau and Thom lai have revealed two fragments of deletion with six and seven nucleotides which were the most identical to the *SalT* reference gene. Two primer pairs have been successfully designed to identify the *SalT* candidate genes in Vietnamese rice landraces. This study provides useful information of salinity tolerance of some Vietnamese rice landraces for breeding programs.

Introduction

Salinity tolerance of rice derives from genes that limit the rate of salt uptake from the soil and the transport of salt across the plant, adjust the ionic and osmotic balance of cells in roots and shoots, and regulate leaf development and onset of senescence [1]. Salinity is a key abiotic constraint devastating crop production in the world. One-fifth of irrigate arable lands in the world has been reported to be adversly affected by high soil salinity. Saline soils occur naturally in both coastal areas, where ground water is contaminated by sea water rise, and in areas subjected to irrigation and/or draining. Salt stress is one of the major serious factors limiting the productivity of rice crop in many worldwide areas. According to report of FAO [2] over 800 million ha of worldwide arable lands are severely salt affected and approximately 20% of irrigated areas. In Asia, 21.5 million hectare of land areas are being influenced by salinity and estimated to cause the loss of up to 50% fertile land by the 21st midcentury [3].

Rice (*Oryza sativa* L.) is the most important food in Vietnam, providing daily food for over 90 million people in this country. Presently, Vietnam is a second biggest rice exporter in the world, accounting for 50% of the world rice trade [4]. Most of Vietnam's rice is produced in the areas of Mekong and Red River Deltas where most acute rice growing areas in close vincity of the sea are vulnerable to salinity. Moreover, this country has been ranked as one of the countries most affected

by climate change, as a consequence, rice production is particularly vulnerable [5]. Salt intrusion due to sea level rise has caused adverse effects on one million cultivated hectares. equally with 3.0% of total areas of this country, causing the economic loss by salt intrusion in 2005 was up to 45 million USD [6]. To date, approximately 600.000 ha of rice growing areas in this country are being severely influenced by drought and saline intrusion in the early year of 2016, has caused seriously economic losses up to 15 trillion VND in rice only (≈ 670 million USD) and reduced rice harvest by up to 50% [7]. Therefore, the need to develop salinity tolerance rice varieties to cope with adverse climate change is very imperative.

To enhance salt tolerance of crops, included rice crop, it undergoes a variety of changes from physiological adaptations to gene expression. There are numberous such as reclamation, irrigation, and drainage are used to minimize soil salinity. However, it is not always shown economical or practical. Based on the known mechanisms of rice plant to response salt stress, the three major approaches to enhance rice salinity tolerance in rice are being utilized: (i) conventional breeding, (ii) molecular breeding and (iii) genetic engineering. Among them, the most feasible methods to develop the rice varieties with salinity tolerance is widely applied via molecular breeding. Salinity tolerance in rice is a complex traits, controlled by the typical at least 5 to 8 significant genes/QTLs which related in both the whole plant level and cellular level, involving interactions of stress with molecular, biochemicals and physiological processes at different stages of plant growth and development [8-9] .In order to initially launch the breeding program, it requires new and accurate genetic sources of this tolerance and more efficient techniques to evaluate the salt tolerance germplasm. Advanced powerful molecular tools, included the complete genome sequence of rice was initially generated in 2005 are available [10], which may be facilitated to find the new tolerant genetic resouces. *SalT* gene is known as one of most reported, co-localizing with the QTL *Saltol* on the chromosome 1 of rice. *SalT* protein was first isolated and chracterized from the roots of salt-treated rice plant by Claes et al [11]. Some previous studies on induction of the *SalT* protein in response to salinity were also reported [12-13]. Numberous QTLs/genes, candidate genes involving in salt tolerance in rice have been so far identified such as *SalT, RSS1, SCK*1, *saltol* etc [1, 10-14]. However, there is no report on identifying SalT gene in Vietnamese rice landraces. Hence, based on the genome sequence databases of 36 Vietnamese rice landraces, the objectives of this study were to evaluate the similarities (homologous segments of DNA and proteins), then designing marker to accurately identify candidate gene *SalT* to further utilize for molecular breeding program to develop salt tolerance rice varieties.

Materials and Methods

Plant Materials

Total 36 Vietnamese rice landraces (Table 1) were kindly provided by Vietnam Plant Resouces Center in 2016, and their genomes were previous re- sequenced [15]. The databases of genome sequence of the rice landraces were used for study.

Table 1. List of 36 genome sequenced Vietnamese rice landraces.

No	Name of landrace	Source	No	Name of landrace	Source
1	Tam xoan Bac Ninh	Bac Ninh	19	Coi ba dat	Khanh Hoa
2	Tam xoan Hai Hau	Nam Dinh	20	OM5629	Vinh Long
3	Te Nuong	NorthVietnam	21	Nep bo hong Hai Duong	Hai Duong
4	Nang thom cho dao	Long An	22	Tan ngan	Yen Bai
5	Thom Lai	Long An	23	Ba cho K'te	Binh Dinh
6	Nep man	Da Nang	24	Blao sinh sai	Hoa Binh
7	Chiem do	Quang Tri	25	Nang quot bien	Bac Lieu
8	Lua Ngoi	Quang Ninh	26	Tep Thai Binh	Thai Binh
9	Mot bui do	Bac Lieu	27	Khau dien lu	Thanh Hoa
10	Nang co do 2	Bac Lieu	28	Nep meo nuong	Lao Cai
11	Ble te lo	Lang Son	29	Toc lun	Hue
12	Chiem nho Bac Ninh 2	Bac Ninh	30	Hom rau	Nam Dinh
13	Nep lun	Ha Giang	31	Nep ong tao	Long An
14	Khau mac buoc	Nghe An	32	OM3536	Vinh Long
15	OM6377	Can Tho	33	Khau Lien	Can Tho
16	Chan thom	Khanh Hoa	34	Lua goc do	NorthVietnam
17	Xuong ga	Tay Ninh	35	Chiem da	Quang Ninh
18	Khau giang	Nghe An	36	IS1.2	AGI

Methods

To predict and analysis of nucleotides, the software NextGENe_V2.3 was used to detect SNPs/InDels, along with the use of phylogenetic software and compare the nucleotide sequence for MEGA6.0 Windows (www.megasoftware.net/mega.php; www.softgenetics.com/NextGENe; http://rice.plantbiology.msu.edu/cgi-in/ORF_infopage.cgi?orf=LOC_Os02g48964).

The method to design the specific primers were conducted as following: Based on the difference of the rice genome sequence, the Primer 3.0 software was used to design the primer sequence (http://primer3plus.com/web_3.0.0/primer3web_input.htm). All the software products are opening sources and available to use as the above links.

Method to test resistance of candidate gene:

Leaf samples of each rice landrace were collected and extracted DNA following the CTAB methods with some modification [16]. PCR reactions were perfomed by Veriti 96-well Thermal cycler. Total volume was 15 µl, included: 5µl DNA; 0.15µM primer; 0.2 mM dNTPs; 1X Buffer PCR; 2.5mM $MgCl_2$ and 0.25 Taq polymerase.

PCR products were performed on electrophoresis on 6% gel polyacrylamide for further analysis.The gels were stained in 0.5 mg/ml ethidium bromide and were documented using Alpha Imager 1220 (Alpha Innotech, CA, USA).

Results and Discussion

Identification of homologous sequence to SalT and nucleotide analysis in CDS (Coding DNA Sequence) and amino acid analysis of candidate gen SalT

The databases of genome sequences of 36 rice landraces were utilized to find homologous sequences of *SalT* gene (salinity tolerance) based on the published GenBank code: Z25811.1 [17]. We have found nine sequence segments which shown equivalent length to the *SalT* reference gene from genome sequences of nine rice landraces: Te Nuong, Khau mac buoc, Chan thom, Khau giang, Tan ngan, Nang thom cho dao, OM5629, Hom rau and Thom Lai. The nucleotide sequences of nine *SalT* homologous genes (*SalT* candidate genes) were analyzed and shown in Table 2. The length of

SalT candidate genes from four landraces: Nang thom cho dao, OM5629, Hom rau, and Thom Lai differed to *SalT* reference gene from one to five nucleotide (Table 2) leading to little different in percentage of T, C, A, and G. While, the five remaining landraces: Te Nuong, Khau mac buoc, Chan thom, Khau giang, Tan ngan have had the similar percentage of four types of nucleotide compared to each other but differed to that of *SalT* reference gene, caused by longer gene sequences (16, or 17bp) of these candidate genes in comparison with sequence of *SalT* reference gene.

Table 2. Nucleotide sequence analysis of *SalT* reference gene and *SalT* candidate genes from nine landraces.

No.	Reference gene and rice landrace name	Nucleotide (%)				Total Nucleotide
		T	C	A	G	
1	*O.sativa salT gene*	29.6	17.9	33.1	19.4	2637
2	Te Nuong	30.0	19.3	30.8	19.9	2654
3	Khau mac buoc	30.0	19.3	30.8	19.9	2653
4	Chan thom	30.0	19.3	30.8	19.9	2653
5	Khau giang	30.0	19.3	30.8	19.9	2653
6	Tan ngan	30.0	19.3	30.8	19.9	2654
7	Nang thom cho dao	29.8	17.9	32.8	19.4	2636
8	OM5629	29.9	17.9	32.7	19.5	2643
9	Hom rau	29.9	18.0	32.6	19.5	2642
10	Thom Lai	29.8	17.9	32.8	19.4	2636

Further analysis of nucleotide sequences in CDS (coding DNA sequence) and amino acid contents of nine candidate genes were shown in Table 3, and Table 4, respectively. These results showed that all candidate genes had similar length in CDS with 438 nucleotides and resemble polypeptide length with 145 amino acids in comparison with that of reference gene. However, the contents of nucleotide in CDS and contents of amino acids of nine candidate genes were shown difference between two groups of landraces. The first group containing four landraces: Nang thom cho dao, OM5629, Hom rau, and Thom Lai, had candidate genes that were akin to the reference gene with both percentage of nucleotide in CDS and amino acid content. While, the second group which contained the rest of landraces had different contents of nucleotide and amino acid (percentage of four amino acids: *His, Leu, Pro,* and *Gln*) when compared to that of *SalT* reference gene. These results imply that the difference in length of nine candidate genes in comparison with reference gene (Table 2) occur in the intron region, which is not effected to the length of CSD and polypeptide.

In last decades, some studies reported that the molecular response of rice to NaCl stress caused by *SalT* and has been weakly expressed in untreated plants, but showed high level protein in sheaths after plants are osmotically changed [11, 14]. Similarly, Benitez et al. [18] reported that *SalT* gene apparently is not directly involved in salinity tolerance by inducing at a higher level in the sensitive varieties. Other report of Teraoka et al. [19] stated that novel mannose-binding (MRL) rice lectin composed of some insolectins and relation to a stress-inducible *SalT* gene, specifically, the cDNA and the genomic clones encoding the MRL 4.85 were highly homologous to the *SalT* gene induced by salt and drought stresses in rice plant in both nucleotide sequences of DNA and the deduced amino acid sequence [20].

Table 3. Analysis of nucleotide in CDS region of *SalT* candidate genes.

No	Name of rice landrace and reference gene	Nucleotide in CDS (%)				Nucleotide in CDS
		T(U)	C	A	G	
1	*O.sativa salT* gene	24.9	22.1	26.0	26.9	438.0
2	Te Nuong	24.0	22.6	26.0	27.4	438.0
3	Khau mac buoc	24.0	22.6	26.0	27.4	438.0
4	Chan thom	24.0	22.6	26.0	27.4	438.0
5	Khau giang	24.0	22.6	26.0	27.4	438.0
6	Tan ngan	24.0	22.6	26.0	27.4	438.0
7	Nang thom cho dao	24.9	22.1	26.0	26.9	438.0
8	OM5629	24.9	22.1	26.0	26.9	438.0
9	Hom rau	24.9	22.1	26.0	26.9	438.0
10	Thom Lai	24.9	22.1	26.0	26.9	438.0

Table 4. Percentage (%) of amino acids of *SalT* candidate genes.

	***O.sativa salT* gene**	**Te Nuong**	**Khau mac buoc**	**Chan thom**	**Khau giang**	**Tan ngan**	**Nang thom cho dao**	**OM 5629**	**Hom rau**	**Thom Lai**
Ala	5.52	5.52	5.52	5.52	5.52	5.52	5.52	5.52	5.52	5.52
Asp	4.83	4.83	4.83	4.83	4.83	4.83	4.83	4.83	4.83	4.83
Glu	4.83	4.83	4.83	4.83	4.83	4.83	4.83	4.83	4.83	4.83
Phe	2.76	2.76	2.76	2.76	2.76	2.76	2.76	2.76	2.76	2.76
Gly	15.86	15.86	15.86	15.86	15.86	15.86	15.86	15.86	15.86	15.86
His	2.76	2.07	2.07	2.07	2.07	2.07	2.76	2.76	2.76	2.76
Ile	11.03	11.03	11.03	11.03	11.03	11.03	11.03	11.03	11.03	11.03
Lys	4.83	4.83	4.83	4.83	4.83	4.83	4.83	4.83	4.83	4.83
Leu	6.21	5.52	5.52	5.52	5.52	5.52	6.21	6.21	6.21	6.21
Met	0.69	0.69	0.69	0.69	0.69	0.69	0.69	0.69	0.69	0.69
Asn	3.45	3.45	3.45	3.45	3.45	3.45	3.45	3.45	3.45	3.45
Pro	4.83	5.52	5.52	5.52	5.52	5.52	4.83	4.83	4.83	4.83
Gln	2.07	2.76	2.76	2.76	2.76	2.76	2.07	2.07	2.07	2.07
Arg	1.38	1.38	1.38	1.38	1.38	1.38	1.38	1.38	1.38	1.38
Ser	8.97	8.97	8.97	8.97	8.97	8.97	8.97	8.97	8.97	8.97
Thr	6.21	6.21	6.21	6.21	6.21	6.21	6.21	6.21	6.21	6.21
Val	7.59	7.59	7.59	7.59	7.59	7.59	7.59	7.59	7.59	7.59
Trp	1.38	1.38	1.38	1.38	1.38	1.38	1.38	1.38	1.38	1.38
Tyr	4.83	4.83	4.83	4.83	4.83	4.83	4.83	4.83	4.83	4.83
Total	145	145	145	145	145	145	145	145	145	145

Designing primers to identify SalT candidate genes

By software analyses, the results of prediction and comparing gene sequences of *SalT* candidate gene from 36 rice genome sequences showed that the frequency of single nucleotide polymorphisms (SNPs) and segment insertion, deletion (InDels) between landraces were rather high. To identify the distinctive features in nucleotide sequences of candidate genes, these nucleotide sequences were aligned and compared with sequence of the reference gene. The result in Fig. 1 showed clearly the difference between two groups of candidate genes. The first group

containing four candidate genes from landraces: Nang thom cho dao, OM5629, Hom rau, and Thom Lai had the similar nucleotide sequence to the reference gene which showed two nucleotide segments due to deletion by six nucleotides and seven nucleotides. While, the candidate genes from five other rice landraces in the second group showed insertion at these two segments, and also had different nucleotide sequence at several positions when compare with the reference gene (Fig.1).

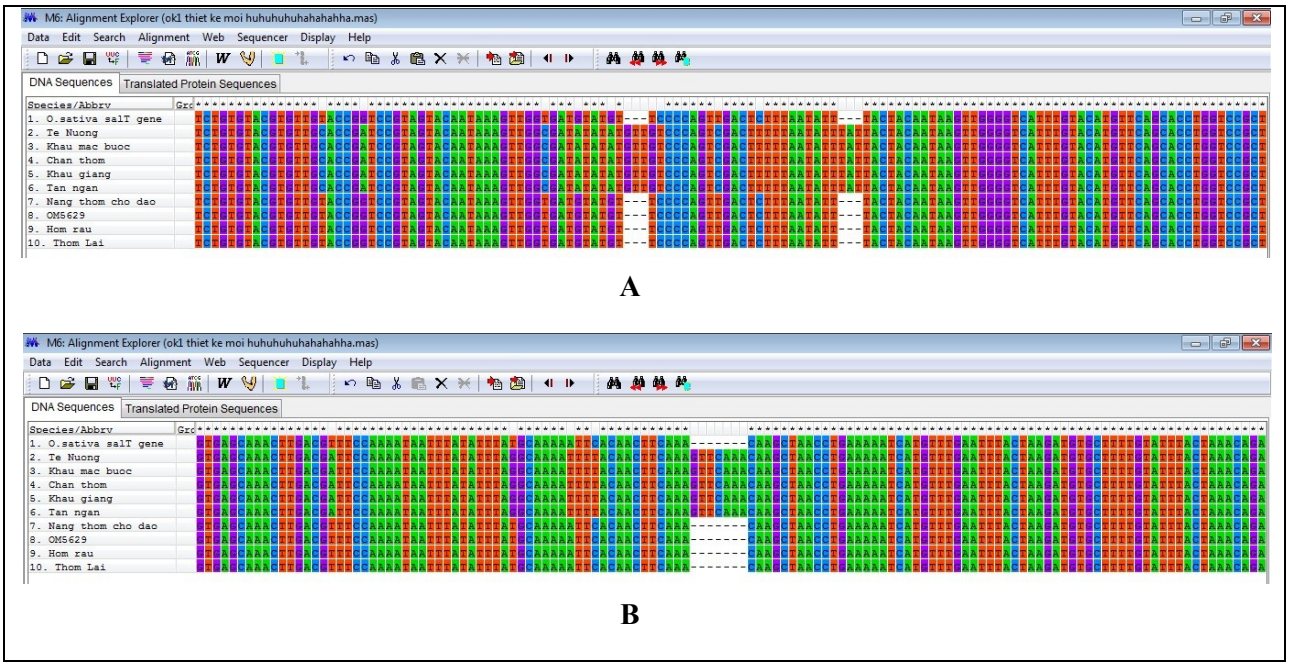

Fig. 1. Nucleotide sequence alignment of *SalT* reference gene and candidate genes from nine rice landraces, (A: 6 nucleotide deletion segment; B: 7 nucleotide deletion segment).

Based on the difference in nucleotide sequence between candidate genes and *SalT* reference gene, two primers namely *SalT1_add7* with the sequence: *SalT1 add7F*: AAACTTGACGATTCCAAAAT/*SalT1add7R*: GTAAATACAAAAGCACATCTT and *SalT1_add6* with the sequence: *SalT1 add6F*: TACGTGTTGCACCGATCCGT/SalT1 add6R: TGCTGAACATGTACAAATGAcc were designed by using Primer 3.0 software. It is able to amplify the candidate gene with the calculated size in the rice landraces carrying the candidate genes involved in salt tolerance, which demontrated identical to the reference gene and different sequence to compare with the published sequence segments (Fig. 2A).

Fig. 2A. Representative images of 6% polyacrylamide gel profile of PCR product from 36 genotypes using *SalT1_add7* primers.

Based on the obtained results of *SalT1_add7* and compared with the published reference gene, it has shown that PCR *SalT1_add7* primers was exposed the band size 116 bp in the landraces among Tam xoan Bac Ninh, Tam xoan Hai Hau, Thom Lai, Nep man, Chiem nho Bac Ninh 2, Nep lun, OM6377, Tep Thai Binh, Toc lun, Lua goc do, Chiem da and IS1.2 landraces. It implies that

those landraces have been similar genotypes with the published sequences. However, the appearance of the band size with 123bp in some landraces indicated the differentiation in comparison with the published sequence of *SalT* gene (Fig.2B).

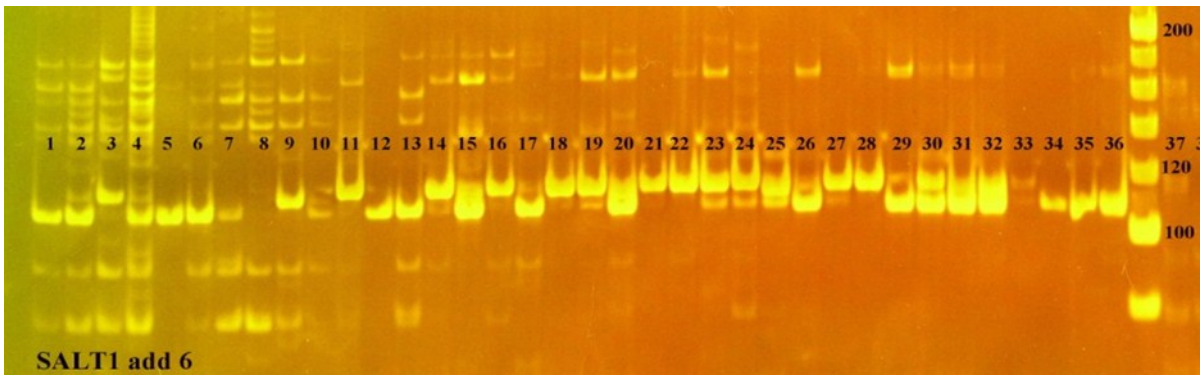

Fig. 2B. Representative images of 6% polyacrylamide gel profile of PCR product from 36 genotypes using *SalT1_add6* primers.

Similarly, by analysis the PCR product of reactions with primer pair *SalT1_add6*, the band size 113bp was found in 20 rice landraces including: Tam xoan Bac Ninh, Tam xoan Hai Hau, Nang thom cho Dao, Thom Lai, Nep man, Chiem do, Mot bui do, Chiem nho Bac Ninh 2, Nep lun, OM6377, Xuong ga, OM5629, Tep Thai Binh, Toc lun, Hom rau, Nep ong tao, OM3536, Lua goc do, Chiem da and IS1.2 landraces. While, the band size 116bp was found in the rest of landraces (Fig.2B). Therefore, these primers should be used to identify the *SalT* candidate genes in the parental materials, progenies to develop salinity tolerance rice lines via breeding program. Recently, the development of salt responsive candidate gene based on the genome sequence databases of plants has been paid much attention. The previous report demonstrated that *SalT* gene is possible to be regulated by ABA-dependant and ABA-independent pathways [14]. In another study reported the evidence of correlation of *SalT* expression with osmoprotectants, such as trehalose and proline, potential role in pathogen agglutination [21-22]. The *SalT* gene is highly expressed in the youngest not fully expanded leaves, but as plants get older, the higher expression levels are found in the oldest leaves of mature plant [14-12]. Hence, the role of *SalT* protein is not limited to environmental stress responses and that it may be included in a broader response/sensor mechanism to the imposed stress [23].

In general, based on appropriate data mining and candidate genes identification, it can help reduce the laborious nature and high cost of both fine-mapping and cloning a large number of genes. It even becomes possible to efficiently process quite large QTL intervals and feasible for using to accelerate the QTL cloning. In crop plant resources, often untapped, the landraces as resources of genetic variation are potential sources of novel salt stress mechanisms and/or QTLs and genes, with their close genetic identity to current crops following candidate genes to be introduced into commercial lines by conventional breeding approaches [24-25]. Molla et al. [26] validated the salt responsive cgSSRs and analyzed their possible to distinguish salt tolerant and susceptible rice genotype. They reported that the repeat length variations in the designed cgSSR loci may play a role in the manifestation of differential behavior of rice genotypes to salt stress. Further functional analysis of the candidate genes within the target region should be then required to determine which gene or genes are responsible for the phenotype. The known candidate genes for salinity tolerance should be introgressed into elite lines and tested in field conditions.

Conclusions

In this study, we have identified nine Vietnamese rice landraces carrying nine *SalT* candidate gene with the sequence similarity of *O.sativa SalT* (the published GenBank: Z25811.1) which have shown salinity tolerance included: Te Nuong, Khau mac buoc, Chan thom, Khau giang, Tan ngan, Nang thom cho dao, OM5629, Hom rau and Thom Lai landraces. Amongst them, four rice

landraces including Nang thom cho dao, OM5629, Hom rau and Thom lai revealed two fragments of deletion with six and seven nucleotides and showed to be similar with the reference *SalT* gene. It has been successfully designed two primer pairs to identify the *SalT* candidate genes in Vietnamese rice landraces for future breeding program.

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