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Gene editing a constitutively active OsRac1 by homologous recombination-based gene targeting induces immune responses in rice

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CONTENTS

Chapter 1

INTRODUCTION

1.1. Plant innate immunity

Plant uses two layers of defense resistance to protect themselves from pathogen infection. The first layer is pattern-triggered immunity (PTI) and the latter is named effector-triggered immunity (ETI) (Zipfel et al., 2005; Chisholm et al., 2006; Jones et al., 2006) (Diagram 1). PTI is initiated by the perception of pathogen-associated molecular patterns (PAMPs) via pattern-recognition receptors (PRRs) located on the cell surface (Zipfel et al., 2008). PAMPs are common feature of pathogens including proteins, carbohydrates, lipids and small molecular such as ATP (John et al., 2006). PRRs fall into two receptor classes: transmembrane receptor like kinases (RLKs) and receptor-like proteins (RLPs) (Dodds et al., 2010). The perception of PAMPs often leads to rapid ion fluxes across the plasma membrane, mitogen-activated protein (MAP) kinase activation, production of reactive-oxygen species (ROS), stimulation of gene expression and cell wall reinforcement (Zipfel et al., 2008). Recently, some PRRs together with their PAMPs have been identified. The best-documented phytobacterial PAMP is flagellin, a component of bacterial flagellum flg22 recognized by receptor Flagellin-sensing 2 (FLS2). In rice, the co-operation of two PRR proteins OsCEBiP, belonged to the group of receptor-like protein, and OsCERK1, belonged to receptor-like kinase is required for the perception of chitin, a main component of fungal cell walls known to induce immune response in various plants (Shimizu et al., 2010). To suppress PTI, bacteria secrete effectors directly into host cytoplasm through type III secretion system (TTSS). Fungal and oomycete pathogens effectors are secreted through the endomembrane system and

subsequently delivered into host cells by poorly discernible mechanism (Kamoun., 2007; Panstruga et al., 2007). Plants counteract effectors by development of resistance (R) proteins, which comprise nucleotide-binding (NB) domain, leucine rich repeats (LRRs) domain, and N-terminus. NB-LRR proteins recognize effector either directly or indirectly through accessory protein, that is a part of NB-LRR complex (Dodds et al., 2010). Similar to PTI responses, ETI includes the generation of ROS, changes in gene expression and activation of MAPKs. However, the most characteristic feature of R gene-mediated defense is the development of the hypersensitive response cell death (Abramovitch et al., 2006). Generally, ETI is quantitatively stronger and faster compared to PTI.

1.2. OsRac1 in plant innate immunity

Rho GTPases belong to the Ras super family of small GTPases, and have been categorized into three sub-families Rho, Rac and Cdc42, based on their sequence homology and cellular functions (Agrawal et al., 2003). Both Cdc42 and Rho present in yeast and animals, while Rac is specific to animal. Plants, however, do not possess any orthologs for any of these fungal or Rho-family GTPase (Yang et al., 2002). Indeed, plants own a unique subfamily of the Rho family, termed Rop (Rho-related GTPase from plant), which slightly shows similarity with mammalian Rac (Agrawal et al., 2003), thereby later is renamed to Rac (also called Rop). There are 7 members of Rac/Rops protein in rice, in which OsRac1 plays a positive role in blast fungus resistance; OsRac4 and OsRac5 are two negative regulators of blast disease resistance. OsRac6 may contribute minor role to blast resistance, whereas OsRac3 and OsRac7 probably play no role in defense (Chen et al., 2010a).

1.2.1. Structure of OsRac1

In general, Rac proteins contain 5 highly conservative domains (G1-G5), insert region and C-terminal region termed hypervariable region (HVR) (Diagram 2). G1, G2 and G3 domains are responsible for GTP hydrolysis. Flexible switch regions I and II in the loops of G2 and G3 are involved in conformation change of Rac proteins, thereby essential for their interaction with downstream effectors and regulatory molecules (Paduch et al., 2001; Vetter et al., 2001). G4 and G5 are known to contact with guanine nucleotide. HVR possesses the polybasic and lipid modification sites functioned to anchor Rac proteins to the plasma membrane. The function of Rho-specific region remains unclear. However, it has recently shown that this region at least involve in the interaction of Rac/Rops with their regulatory guanine nucleotide exchange factor (GEFs) (Thomas et al., 2007). Based on the sequence of C-terminal region, Rac/Rops are divided into two groups. Group 1 contains a conserved CaaL (a, aliphatic acid) motif, whereas group 2 possesses a cysteine-containing element for membrane anchoring (Chen et al., 2010a). OsRac1 belongs to group 2 according to this way of classification.

1.2.2. Regulators of OsRac1

Like the other members in Rac/Rop family, OsRac1 acts as a molecular switch by cycling between inactive (GDP-bound) and active (GTP-bound) form (Van Aelst et al., 1997). The binding of GEFs with small GTPase enables the exchange of GDP for GTP to generate the activated form, which is then capable to recognize downstream targets. In contrast, GTPase activating proteins (GAPs) enhance the endogenous GTPase activity of OsRac1 that turn it back to the inactive state (Diagram 3). Early studies showed that the G19V point mutation in the G1 box domain of OsRac1 with the substitution of Glycine

(GGC) by Valine (GTC) (Diagram 2), corresponding to the G12V mutant in human Rac changed the conformation of OsRac1, resulting in reduced hydrolysis activity. Consequently, OsRac1 protein exists in a constitutively active form (CA-OsRac1) (Kawasaki et al., 1999).

Guanine nucleotide dissociation inhibitors (GDIs) extract the inactivated proteins from the membrane and inhibit the exchange of GDP for GTP and, thus predominantly act as negative regulators for these GTPases (Diagram 3) (Nibau et al., 2006).

1.2.3. The function of CA-OsRac1 in rice innate immunity

CA-OsRac1 functions as an important molecular switch in rice immunity (Diagram 4). The upstream signals of OsRac1 include resistance (R) protein Pit and Gα subunit of trimetric G proteins. CA-OsRac1 interacts with Pit, an NLR family R protein and is required for Pit-mediated immune response such as ROS production and Hypersensitive Response (Kawano et al., 2010). CA-OsRac1 in *d1* (d for dwarf) mutant, which lacks a single copy of Gα gene and addressed the function of G protein in disease resistance, recover SE-mediated defense signaling and resistance to rice blast fungus, suggesting that OsRac1 is located downstream of Gα (Kawasaki et al., 1999).

Plants protect themselves from pathogen infection by a various chemical and physical barriers termed Hypersensitive Response. During this process, recognition of a pathogen triggers the activation of a cell death pathway, resulting in the formation of a zone of dead cells (necrosis) around the site of infection (Koga et al., 1998). Hypersensitive response-like cell death is characterized by the appearance of granule and auto-fluorescence (Koga et al., 1994). Hypersensitive response is accompanied by the induction of pathogen-related genes, the formation of lignin and a burst of reactive

oxygen species (ROS) (Koga et al., 1998). We found that OsRac1 plays a critical role in ROS production and cell death in plant (Kawasaki et al., 1999). NADPH oxidases (Rhoh: respiratory burst oxidase homolog) possess a conserved N-terminal extension that contains two Ca^{2+} binding EF-hand motifs. In 2007 we reported the direct interaction between OsRac1 and N-terminal extension including EF-hand motifs is required for ROS production (Wong et al., 2007). Furthermore, we solved the 3D structure of OsRac1 and RbohB complex, supporting the role of OsRac1 in ROS production (Oda et al., 2010). Furthermore, OsRac1 was found to bind cinnamoyl CoA reductase, an enzyme involved in lignin biosynthesis (Kawasaki et al., 2006), leading to enzymatic activation and increase in ROS production. Thus, it is likely that OsRac1 controls lignin synthesis through the regulation of both NADPH and OsCCR1 activities during defense responses.

The accumulation of pathogen-related (PR) genes in plants in response to pathogens is one of the crucial components in a repertoire of plant's self-defense mechanisms, and widely used as marker genes to study plant's defense mechanism (Jwa et al., 2006). OsRac1 was found to induce the expression of pathogen-related gene Probenazole induced (*PBZ1*) gene upon chitin treatment in cell culture (Thao et al., 2007, Akamatsu et al., 2013). The enhanced accumulation of *PBZ1* was previously observed by inoculation with rice blast fungus, but not by wounding, suggesting its important function in rice disease resistance responses (Midoh et al., 1996). In addition, Phenylalanine ammonia-lyase (PAL) catalyzing the first step in the biosynthesis of phenylpropanoids, was one the first identified plant "defense gene" and was induced by both pathogen and environmental stress. *PAL* is encoded by multi-member family genes and at least some of them are induced by fungal elicitor or fungal infection of plant tissues (Wanner et al., 1995). CA-OsRac1 was demonstrated to interact with OsMPK3 and OsMPK6 via the

activation of OsMKK4. Subsequently, the activated OsMPK3 and OsMPK6 interact with transcription factor RAI1 to regulate the expression of downstream pathogen-related genes *PAL1* and *OsWRKY19* (Lierberherr et al., 2005, Kim et al., 2012).

Recently, we demonstrated that OsRac1 forms a protein complex termed defensome network comprised of heat shock proteins HSP70, HSP90, co-chaperone Hop/Sti1a, scaffold protein OsRACK1, OsRacGEF and OsCERK1 (Thao et al., 2007, Nakashima et al., 2008, Chen et al., 2010b, Akamatsu et al., 2013) at the plasma membrane. Upon chitin perception, OsCERK1 kinase phosphorylates S549 of OsRacGEF1, which activates OsRac1. Activated OsRac1 induces various downstream events in immune responses (Akamatsu et al., 2013). Collectively, CA-OsRac1 function as an important molecular switch in rice immunity.

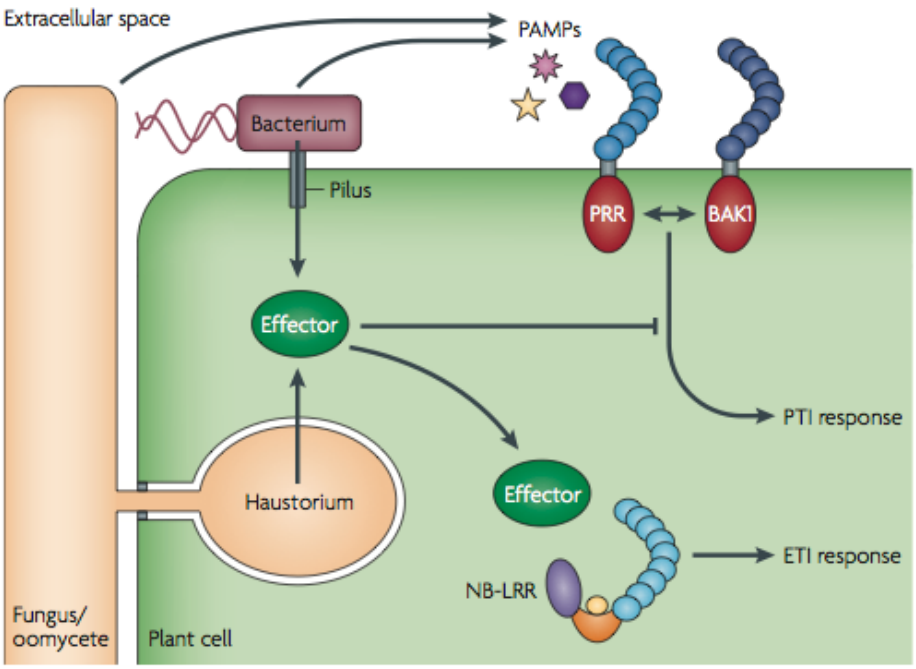


Diagram 1: The principle of plant immunity. Molecules released from the pathogens into the extracellular spaces, such as lipopolysaccharides, flagellin and chitin (PAMPs) are recognized by cell surface pattern recognition receptors (PRRs) and elicit PTI. PRRs consist of an extracellular leucine-rich repeat (LRR) domain (mid blue), and an intracelular kinase domain (red). Bacterial pathogens deliver effector proteins into the host cell by type III secretion pilus, whereas fungi and oomycetes deliver effectors from haustoria or other intracellular structure by an unknown mechanism. These intracellular effectors often act to suppress PTI. However, many are recognized by intracellular nucleotide-binding (NB)-LRR receptors, which induces ETI. NB-LRR proteins consist of a carboxyl-terminal LRR domain (light blue), a central NB domain (orange crescent) that binds ATP or ADP (yellow oval), and an amino-terminal Toll, interleukin-1 receptor, resistance protein (TIR) or coiled-coil (CC) domain (purple oval) (Figure of Dodds et al., 2010)

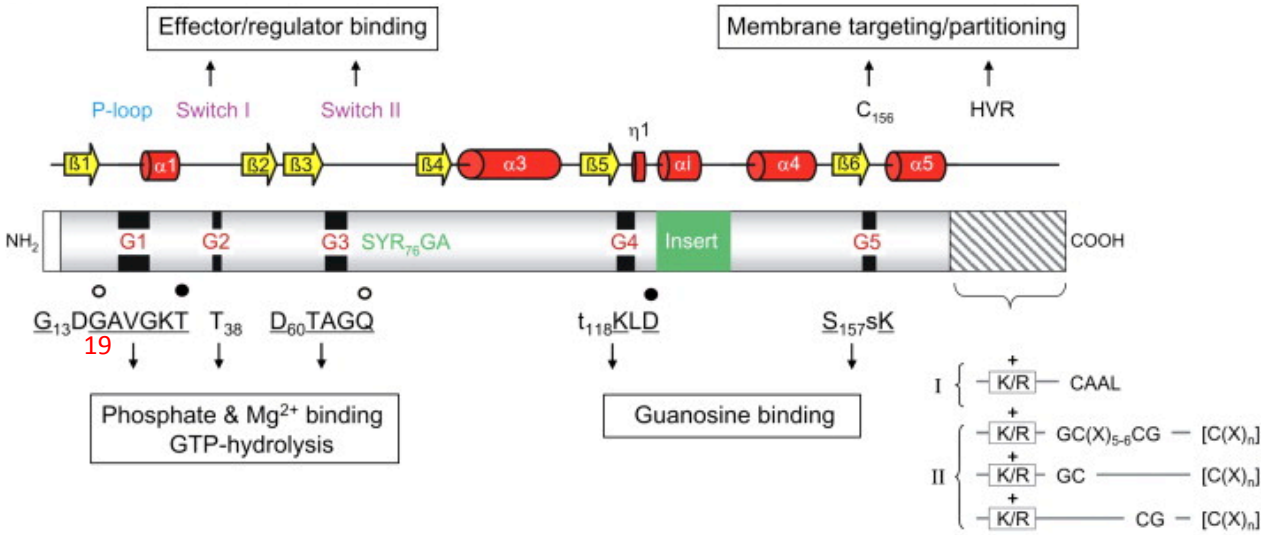


Diagram 2: Structure features of Rac/Rop proteins and their assigned function. Typically, Rac/Rop proteins consist of G domains (grey) G1-G5), Rho insert (green) and C-terminal hypervariable region (HVR: stripy). The consensus sequences of G box motifs are shown with capital letters, in which open circles: commonly mutated amino acids in constitutively active Rac/Rops; filled circles: mutated residues in dominant-negative ROPs. The distinct structures HVR of type I and type II Rac/Rops are given including: K/R box: multiple positively charged K and R residues) and variation of the consensus motifs GC-CG and CaaL (Modified from the figure of Berken et al., 2008).

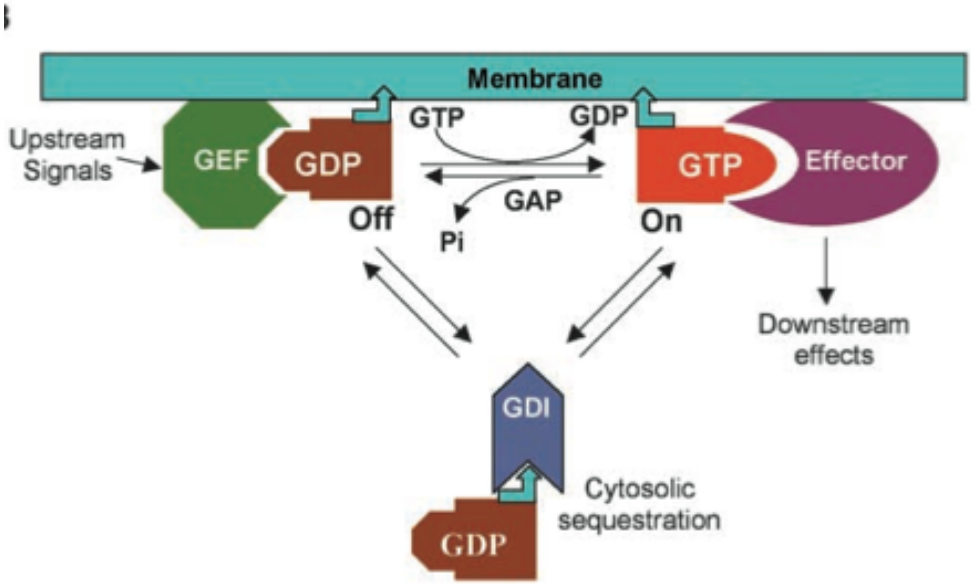


Diagram 3: Rac/Rop activity is regulated via the shuttling between the inactive GDP-bound form and the activated GTP-bound form. Guanine exchange factors (GEFs) stimulate GDP-GTP exchange and activate Rac/Rops on the plasma membrane. GTPase activating proteins (GAPs) stimulate the intrinsic GTPase activity of Rac/Rops, turning it back to the inactivated GDP-bound form. Activated Rac/Rops regulate diverse cellular, biochemical and molecular processes to control the activity of multiple target response system (Figure of Yang et al., 2002)

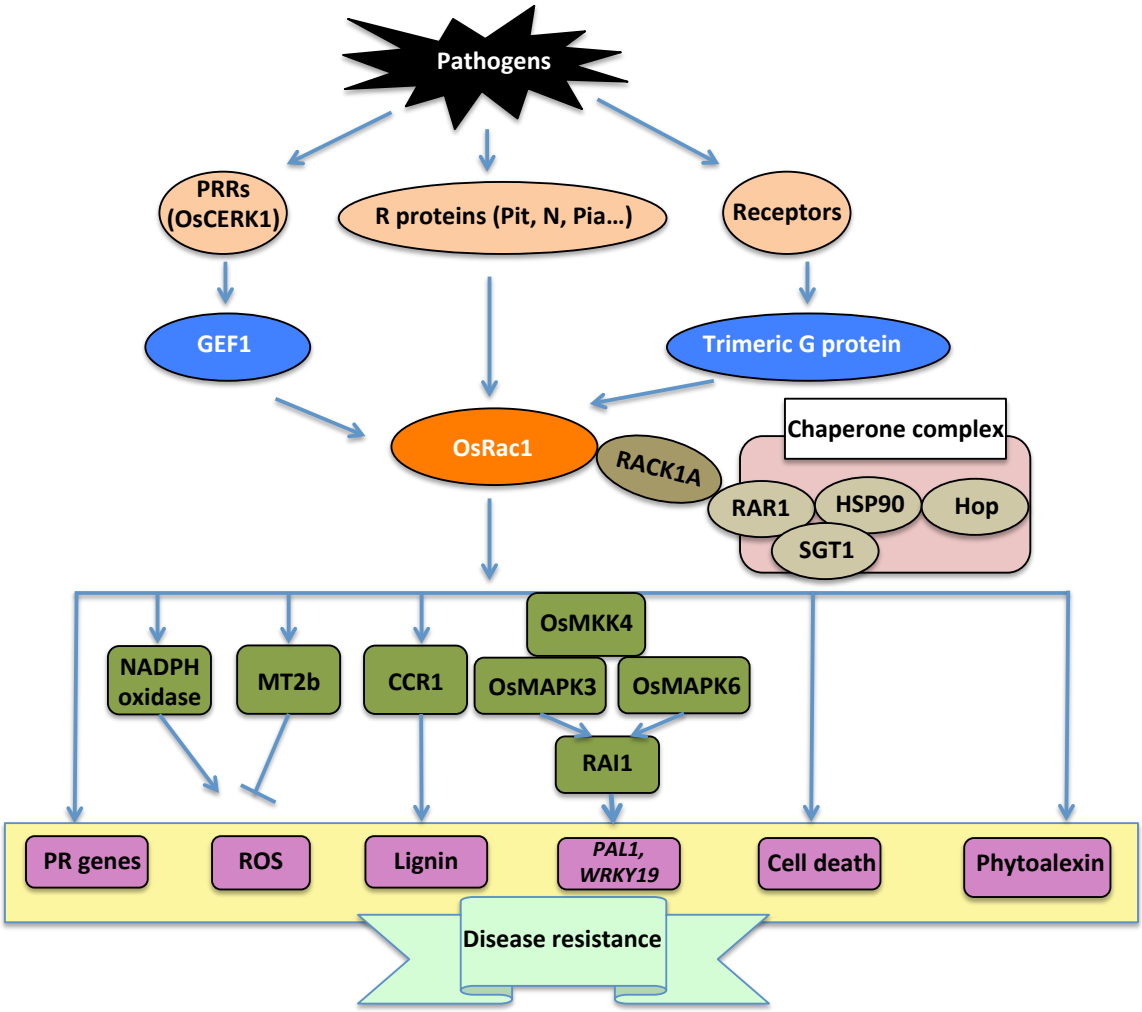


Diagram 4: OsRac1 plays a critical role in immune response. OsRac1 induces various downstream events in immune responses including activation of pathogen related (PR) genes, ROS production, lignin synthesis and interaction with MAP kinase in defense response. OsRac1 forms a protein network called defensome network that comprises heat shock protein HSP90, SGT1, scaffold protein RACK1a, OsCERK1 and GEF1. (Kawasaki et al., 1999; Ono et al., 2001; Lierbereher et al., ; Thao et al., 2007; Nakashima et al., 2008; Chen et al., 2010a, b; Kawano et al., 2010; Kim et al., 2012; Wong et al., 2007, Akamatsu et al., 2013)

1.3. Homologous recombination-based gene targeting and Cre-*loxP* recombination system

1.3.1. Homologous recombination-based gene targeting with positive-negative selection

Homologous recombination (HR)-mediated gene targeting, a powerful tool of reverse genetics (Terada et al., 2002) enables to convert specific DNA sequence in an endogenous gene to a designed sequence through gene replacement (Jhojuka-Hisatomi et al., 2008**)**. In higher plant, the frequency of homologous recombination is often very low due to the overwhelming occurrence of random integration events. This becomes the main obstacle for the development of an efficient homologous recombination-mediated gene targeting method. To improve the efficiency of gene targeting in plants, various approaches have been attempted to increase the homologous recombination and to reduce the non-homologous end joining. One of the strategies focuses on engineering the host recombination and/or repair system in tobacco and Arabidopsis such as modification of proteins involved in HR (DNA repair and recombination Rad54 protein., Shaked et al., 2005), remodeling of chromatin structure (chromatin remodeling INO80 protein, Fritsch et al., 2004; histone binding CAF-1 protein, Endo et al., 2006). However, alteration of the recombination or repair systems often lead to the undesirable phenotypic changes that may hamper the functional characterization of an endogenous gene by targeted modification (Iida et al., 2005). Recently, to increase the efficiency of gene targeting the induction of double strand breaks (DSBs) at the targeted sites by zinc finger nucleases (ZFNs), followed by homologous recombination was applied to modify endogenous loci in the plants of the crop species Zea mays (Shukla et al., 2009). Although this new technology is promising tool for editing plant genes in the future (Zhang et al., 2013),

further studies must be conducted with other plant species before this method can be routinely used. Another approach bases on the specific phenotype of transgenic plants to select true gene targeting among various random integration events. This method was recruited to target *acetolactate synthase* and *anthranilate synthase* genes in rice (Endo et al., 2007, Saika et al., 2011). The latter was altered to gain resistance to an herbicide and the acquired resistance was used for selection of transgenic rice cells. However, this method is limited to some genes encoding specific phenotypes in plants and not always applicable for all the cases.

The strong positive-negative selection applied the first time on rice *Waxy* gene (Terada et al., 2002) becomes a breakthrough to effectively screen true gene targeting (TGT) among various random integration events, opening the ability to modify any genes of interest. In positive-negative selection, positive selection marker located within the segments homologous to the target gene to ensure the foreign DNA is introduced into genome by HR or random integration and the negative selection marker placed at both ends of transformed vectors to select the true HR against the random or non-targeted events that are removed by the toxic effect of negative selection marker (Terada et al., 2007) (Diagram 5). So far, in higher plants, positive marker genes include kanamycin antibiotic resistance gene, β-glucuronidase histochemical marker, purkernels natural pigment genes in maize, and CaMV viral genome (Chittela et al., 2010). The *codA* and *DT-A* genes have been used as negative selection markers in this selection system. The *DT-A* gene, derived from *tox* gene carried by temperate corynephages of *Corynebacteria diphtheriae*, encodes the diphtheria toxin A fragment, causes the NAD^+-dependent ADP-ribosylation of elongation factor 2 and inhibits protein biosynthesis (Pappenheimer., 1977). Recently, Homologous recombination-based gene targeting with strong positive-

negative selection has been broadly applicable to generate knock out, knock in mutants in several important rice genes (Terada et al., 2007, Yamauchi et al., 2009, Moritoh et al., 2012, Ono et al., 2012, Ozawa et al., 2012). In 2008, various nucleotide substitutions in the *Adh2* locus were generated by employing this method (Jhozuka-Hisatomi et al., 2008).

1.3.2. Cre-*loxP* site specific recombination for marker free

To establish genetically modified organism (GMO), the antibiotic-resistance marker genes are required to be removed from gene targeting lines because they might transfer to the pathogens or closely related weed species (Sreekala et al., 2005). Cre-*loxP*-site specific recombination has been employed to eliminate selection marker. The Cre protein (38kDa) is encoded by the *E. coli* phage P1. P1 is maintained inside *E. coli* cells as a single copy, circular DNA plasmid molecule. The role of Cre protein is to exchange and separate copies of P1 that arises after its replication in order to allow partitioning of the two P1 molecules at each cell division (Abremski et al., 1984; Sternberg et al., 1981). The target site of Cre is the *loxP* sequence of 34 bp, containing an 8 bp core sequence flanked by two 13 bp inverted repeats. Two Cre molecules bind to each *loxP* site, one on each half of palindrome (Van Duyne., 2001). Cre molecules bind to DNA then form a tetrameric complex, bringing two *loxP* sites into proximity, and cleaving the fragment flanked by two *loxP* sites. Consequently, single *loxP* site is left on the original sequence.

Taking advantage of Cre-*loxP*-site-specific recombination system, marker is designed to be flanked by two directly repeat *loxP* sites so that under Cre activity, the precise recombination and excision of two *loxP* sites enable the elimination of intervening sequence including marker gene (Dale et al., 1999) (Diagram 6). Several

attempts have been employed to introduce Cre into transgenic plants including sexual cross (Odell et al., 1990), re-transformation (Gleave et al., 1999) or delivery of Cre fused with nuclear localization signal (*N*Cre) into rice plant by particle bombardment (Terada et al., 2010). Those methods are recognized to cause toxic effect for the cell because of continuous exposition to genomic DNA (Terada et al., 2010). To overcome this, chemical-inducible Cre has been recruited to suppress its activity until the accomplishment of the selection step (Zuo et al., 2001; Hare et al., 2001; Zhang et al., 2003; Sreekala et al., 2005). Under chemical inducer, Cre expression enables the splicing of DNA fragment comprising *Cre* gene itself and selectable marker flanked by two *loxP* sites (Sreekala et al., 2005)

1.4. Thesis rationale

Previously, CA-OsRac1 was generated by introducing a G19V point mutation in *OsRac1* gene that was fused to the CaMV35S promoter and transforming rice with this construct (Kawasaki et al., 1999). Functioning of the CA-OsRac1 mutation created in this system largely depends on random integration of the designed T-DNA, and the constitutively expressed CaMV35S promoter caused some undesirable effects on plant growth including dwarfing and sterility. Therefore, to investigate further the effect of CA-OsRac1 on rice immunity and to establish rice plant enhanced disease resistance for crop improvement, we used homologous recombination-based gene targeting (GT) to produce transgenic rice plant whose endogenous OsRac1 locus is modified to the CA form and is expressed under the control of the native promoter.

To generate G19V mutation, two gene targeting vectors containing point mutation, *hpt* positive selection marker inserted into the first or the third intron of *OsRac1* and DT-

A positive selection marker placed at both end of vectors were transformed into wild type rice to introduce G19V mutation by homologous recombination. True homologous recombination events containing G19V point mutation and integration of *hpt* marker were selected by positive-negative selection combined with PCR and sequencing. Further more, the *hpt* positive selection marker was removed from the rice genome by using Cre-*loxP* recombination system, giving marker free G19V transgenic rice plants. The CA-OsRac1 generated by gene targeting was termed CA-gOsRac1. The analysis of resultant *CA-gOsRac1* plants revealed that the CA mutation was effectively transferred from vectors to *OsRac1* locus and stably transmitted to the next generation. Transgenic plants showed enhanced immune responses compared to wild type plants, although the expression of homozygous *CA-gOsRac1* was suppressed at the mRNA level, for unknown reasons.

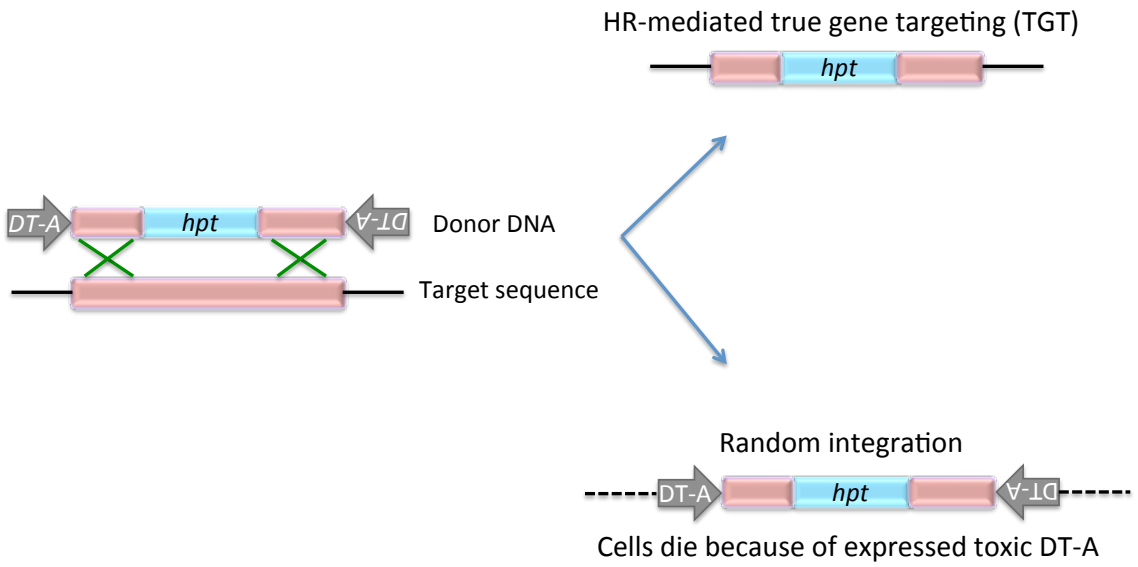


Diagram 5: Positive-negative selection. Positive selection marker for hygromycin resistance located between the homologous segments to ensure the introduction of foreign DNA either by homologous recombination (HR) or random integration (RI). Negative selection markers encoding diphtheria toxin placed at both ends of transferred vector to choose homologous recombination events among random integration events. RI with the expression of DT-A protein causes the cell death (Modified from figure of Terada et al., 2007)

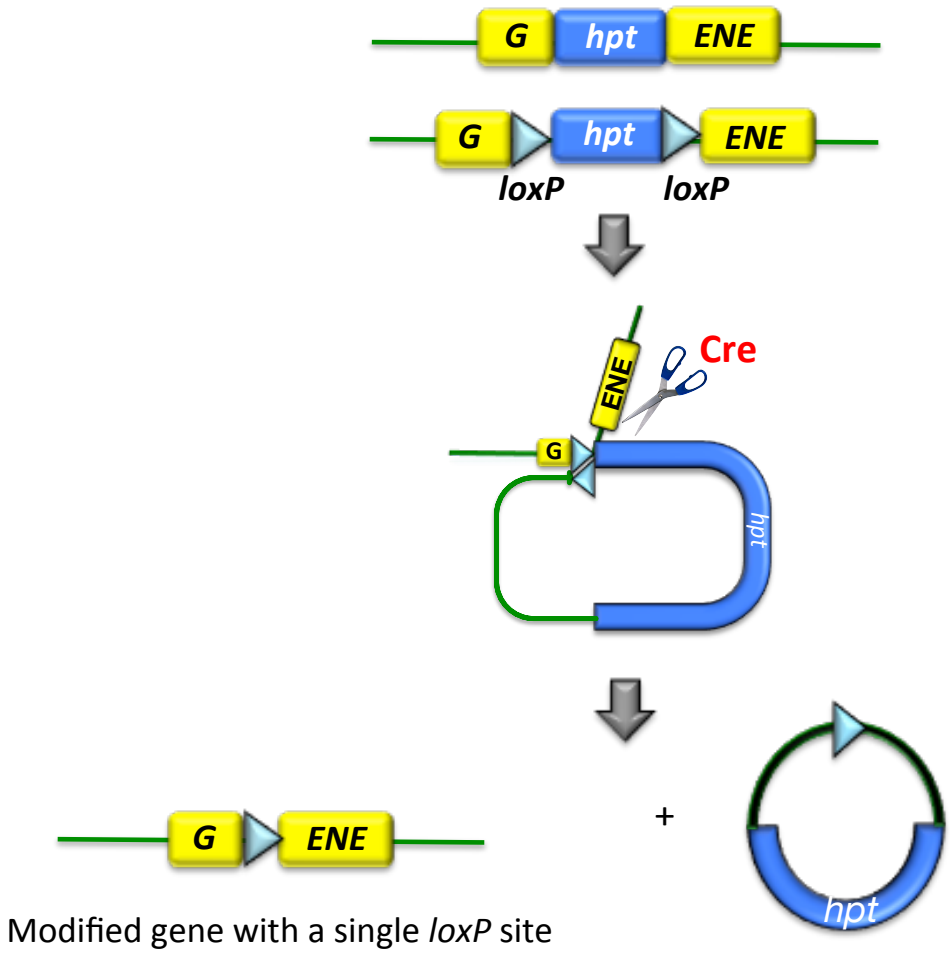


Diagram 6: Cre-*loxP*-site specific recombination for marker free. Cre protein is driven by exogenous promoter and binds to *loxP* sites. *LoxP* sites are brought close proximity and recombine, leading to the elimination of *hpt* gene. Modified gene shows the presence of a single *loxP* site. (Modified from the figure of Smith et al., 2011)

Chapter 2

MATERIALS AND METHODS

2.1. Materials and methods for generating OsRac1-G19V mutation

2.1.1. Nucleic acid procedures

General nucleic acid procedures, including plasmid preparation, plant DNA isolation, PCR amplification and DNA sequencing analysis were described previously (Terada et al., 2002; Moritoh et al., 2012).

2.1.2. Construction of Plasmid vectors

For the construction of gene targeting vectors, vector A and vector B, 5' and 3' homologous regions (Fig. 1A, Fig. 2A) were prepared from the Kinmaze genome by nested PCR amplification using appropriate primer sets (Supplementary Table S1) and PrimeStar GXL polymerase (Takara bio, http://www.takara-bio.com/). Resultant fragments were cloned into pCR-BluntII-TOPO or pCR4Blunt-TOPO using ZeroBlunt TOPO PCR Cloning Kit (Life technologies, http://www.lifetechnologies.com/). Subsequently, the clones harboring 5' homologous regions for vector A or vector B were subjected to introduce G56T nucleotide substitution to corresponding site of the exon1 in *OsRac1* gene that causes G19V substitution in OsRac1 protein using Quick Change II XL Site-Directed Mutagenesis Kit (Agilent technologies, http://www.home.agilent.com/) with the primer sets shown in Supplementary Table S1. Then homologous regions for vector A and vector B were built into *Pac*I-*Srf*I and *Pme*I-*Asc*I cloning sites of pINA134 (Terada et al., 2002), respectively. DNA sequencing analysis of homology regions

showed no unexpected base changes caused in cloning steps. In order to optimize PCR screening conditions for detection of authentic homologous recombination at *OsRac1* locus, the control vectors carrying predicted genome structure of targeted locus, were constructed in the same way by cloning longer homologous regions containing A-5JF/A-3JF or B-5JF/B-3JF.

For transformation in order to introduce ß-estradiol inducible XVE-Cre/*loxP* system into targeted calli, *hygromycin phosphotransferase* gene (*hpt*) in pBIMFN (Nishizawa et al., 2006) was replaced to *neomycin phosphotransferase gene II* (*nptII*) through homologous recombination in *E. Coli* (Chaveroche et al., 2000, Zhang et al., 2000). The resulting vector was designated pBIMFN-*nptII*.

2.1.3. Plant transformation and β-estradiol treatment for Cre-loxP-site specific recombination.

Japonica rice cv. Kinmaze (*Oryza sativa L.*) was used for plant material. Process of gene targeting by large-scale *Agrobacterium*-mediated transformation was according to previous reports (Terada et al., 2002, 2007). PCR screenings for targeted lines with appropriate primers (Figure 1; Supplementary Table S1) were carried out using Prime Star GXL DNA polymerase (Takara bio). The thermal cycles of PCR reaction were as follows: an initial denaturation at 98˚C for 30 s; 40 cycles of 98˚C for 10 s, 61˚C for 30 s, 68˚C for 10 min; and a final extension at 68˚C for 10min. Amplified DNA fragments obtained from targeted lines were subjected to sequencing analysis to confirm the structure of targeted locus generated HRs with no mutations. The targeted lines carrying G56T substitution in exon1 of *OsRac1* gene were individually transformed with pBIMFN-*nptII* by conventional method (Hiei et al., 1994). In order to eliminate *hpt*-

Δ*En*/*Spm* fragment from targeted *OsRac1* locus through Cre-*loxP* site-specific recombination, paromomycin (40 mg L^{-1}) resistant callus lines were treated with ß-estradiol for 4 to 7 days according to Usuda et al (2009). Plants without the *hpt*-Δ*En*/*Spm* fragment arose from the callus lines were selected by PCR analysis with the primer pairs, ACL-F/ACL-R or BCL-F/BCL-R (Fig. 1C, Fig. 2C, Supplementary Table S1). The DNA fragments amplified by PCR with the primer sets, 5-1st-F/5-1st-R2 and 3-1st-F/5-1st-R2 were purified with MonoFas DNA Purification Kit I (GL Sciences, http://www.glsciences.com/) and directly sequenced with the primers shown in Supplementary Table S1. Obtained fragments were also cloned into pCR4Blunt-TOPO using ZeroBlunt TOPO PCR Cloning Kit for sequencing (Life technologies), and the clones carrying single *loxP* site were selected with the primer set, H1F/5-1st-R2 (Supplementary Table S1). The DNA sequence of consecutively modified region through GT and Cre-*loxP* site-specific recombination was analyzed with the appropriate primers.

2.2. Materials and methods for identifying characterizations of *CA-gOsRac1*

2.2.1. Cell cultures and fungus growth condition

Rice cell culture expressing *CA-gOsRac1* was generated from seeds and maintained in R2S medium according to previous method (Kawasaki et al 1999., Ono et al., 2001). Rice seeds were immersed and shaken in 70% ethanol for 1 min, then in 10% solution of bleaching powder for 10 min. After being washed thoroughly, the sterilized seeds were put on N6 media [4 g N6 basal salt (Sigma C1416), 1 ml 1,000 x N6 vitamin, 30 g sucrose, 100 µl 2,4-D 20 mg/ml, resolved in H_2O up to 1 l]. After 3 weeks at 27^0C, the callus induced from the germinates were transferred on the agar and successively sub-

cultured at least ten times at intervals of 2 or 3 weeks, then maintained in liquid R2S media (50 ml R2S macro, 1 ml R2S micro, 1 ml MS vitamin, 2 ml Fe-EDTA, 100 µl 2,4-D, 30 g sucrose, adjusted pH5.6-5.8 and supplemented H_2O up to 1 l). The growth condition of rice blast fungus race 007, compatible with cv. Kinmaze was described by protocol of Takahashi et al (1999), Koga et al (1994). The rice blast fungus was grown on oatmeal agar medium (30 g l^{-1} oatmeal, 5 g l^{-1} sucrose, and 16 g l^{-1} agar) at 22^0C. The conidia used for inoculation were produced by maintaining cultures on agar medium under fluorescence light for 3 days after brushing the aerial hyphae with water.

2.2.2. Chitin treatment and quantitative real-time PCR

0.5 mg cell cultures were treated with 20 µg/ml hexa-N-acetylchitohexaose (Sigma) and harvested at indicated time points. Total mRNA was extracted by Trizol method (Invitrogen), followed by DNaseI treatment (Invitrogen). cDNA was synthesized from 1 µg of total mRNA by oligo-dT primer. Expression of *OsRac1*, *PAL1* and *PBZ1* was monitored by quantitative real time PCR using SYB Green (Applied Bio-system). Data was collected from ABI PRISM 7000 detection system according to manufacturer's instructions. *Ubiquitin* was used as an internal control. The primer sequences are listed in Supplementary Table S1.

2.2.3. Infection assay and trypan blue staining

Infection assay in leaf sheath

One month-old plants were infected with rice blast fungus compatible race 007 by intact injection method (Koga et al., 2004). Spores were induced by maintaining fungal plate under UV light for three days after brushing the surface by water. In the next step,

spores were purified by filter paper. Gently, intact leaf sheath was laid horizontally, filled with 20 µl fungal spore ($1X10^5$ conidia/ml) and incubated at 25^oC in dark, high humidity chamber for 24 h. At each infection site, leaf sheath was sliced into thin layers to observe under microscope with bright field and fluorescence light. Pictures were taken 72 hr after infection. Trypan blue staining in rice leaf was a little bit modified from protocol of Yin et al (2000). Leaf sheath was spliced, submerged in 70^oC trypan blue solution (2.5 mg/ml trypan blue, 25% wt/vol lactic acid, 25% water saturated phenol, 25% glycerol and water) for 10 minutes, then washed with water to remove excess staining. Next, samples were kept overnight in de-staining solution (acetic acid: ethanol =1:3), then washed again by 70% ethanol for 2-3 days and submerged in water before microscope observation.

Infection assay and DNA-based real time PCR in leaf blade

The two youngest leaf blades of one-month old plants were selected for infection by punch infection method (Chen et al., 2010a). Lesion lengths were measured 5 days (compatible race) and 12 days (incompatible race) after inoculation. The infection ratio was calculated based on *M. oryzae* and rice DNA using specific primers against *M. oryzae Pot2* and rice *Ubiquitin* as shown in Supplementary Table S1 (Berruyer et al., 2006). The *Pot2* transposon sequence used to quantify the fungus has ~ 100 copies in the *M. oryzae* genome. An infection ratio (*MgPot2*/*Ubiquitin* x 100) represents the relative number of fungus cells per plant cell.

2.2.4 Microarray analysis

Total mRNA was extracted from leaf sheath of transgenic plants by Trizol method (Invitrogen). cDNA was synthesized by oligo-dT T7 promoter primer and reverse transcriptase. cRNAs were generated and labeled with Cyanine3 CTP (Cy3) and

Cyanine5 CTP (Cy5) using T7 RNA polymerase. The next stage includes the purification and OD measurement of labeled cRNAs. For the hybridization step, first 825 ng labeled cRNAs were fragmentized by incubating with fragmentation buffer at 60^{o}C, 30 min in dark, then adjusted to a final volume of 110 µl before applying to the Aglient 44K microarray array slides for incubation at 65^{o}C, 17 hours, 10 rpm. After hybridization, slide was removed from chamber while immersed in wash buffer 1 and gently washed at room temperature, 1 min. Upon gently washing at 37^{0}C, 1 min in wash buffer 2, slide was removed slowly from wash box and sprayed with N_2 gas to remove dust particles. Finally, the slide was set in scanner holder for scanning according to manufacture's instruction.

In this study, two lines from homozygous plants were analyzed (2 biological replicates). Each biological replicate was repeated three times. The raw data was imported to Subio Basic Plug-in software (http://www.subio.jp/products/basicplugin) and filtered by signals intensities. Differently expressed transcripts were calculated based on the cut off value of two, P value < 0.05.

2.2.5. Northern blotting assay

Total RNA was extracted from the leaf sheath of one month-old plants using RNeasy Plant Mini Kit (Qiagen; http://www.qiagen.com). A number of 30 µl of total RNA (500 ng/µl) was loaded and separated on 1.2% formaldehyde gel, and transferred onto Hybond-N membrane (http://www.gelifesciences.com). The probe was synthesized based on random primer DNA labeling kit (http://www.takara.co.kr). The sequences of primers to amplify DNA template for probe synthesis are shown in the Supplementary Table S1. Hybridization was performed at 65^{0}C, and the washing steps were performed

under high-stringency condition at 65^0C.

2.2.6. Bisulphite sequencing

Bisulphite sequencing was modified from protocol of Okano et al (2008) using MethylEasy™ *Xceed* Kit (www.geneticsignatures.com). Genomic DNA was extracted from leaf blade and purified by Mini Elute PCR purification Kit (Qiagen) followed by bisulphite treatment. Nested PCR was applied to amplify 1 kb promoter fragment and 400-bp gene body region surrounding *loxP* site by primer sets shown in Supplementary Table S1. PCR products were cloned into pCR-Blunt II-TOPO (Invitrogen) vector and sequenced using Big-Dye terminator cycle sequencing Kit and an ABI 3100 sequencer, corresponding to standard protocol. In this experiment, we assayed 3 plants per background (homozygous and wild type), and sequenced 16 colonies from each plant. The percentage of methylation was calculated by the formula: (the number of methylated colonies/the total number of sequenced colonies) x 100%

Chapter 3

RESULTS

3.1. Generation of single nucleotide substitution in *OsRac1*

The substitution of Glycine (GGC) to Valine (GTC) in the amino acid 19 of OsRac1 protein sequence (G19V) is corresponding to the substitution of G to T in the nucleotide 56 of *OsRac1* gene (G56T). This point mutation was introduced by gene targeting (GT) with positive-negative selection, followed by the elimination of inserted *hpt* positive marker in *OsRac1* locus by Cre-*loxP* mediated recombination (Fig. 1, Fig. 2). To control the induction of Cre-*loxP* recombination, XVE-Cre-*loxP* system that is shown to be activated by ß-estradiol (pBIMFN, Usuda et al., 2009) was employed. GT vector was designed to leave single *loxP* site in the first or third intron of *OsRac1* locus in the resultant transgenic plants (Fig.1C, Fig. 2C).

3.1.1. Construction of targeting vectors

The GT vectors, vector A and vector B were designed to introduce G56T nucleotide substitution set in their 5' homology regions into the first exon of *OsRac1* gene and positive selection marker *hpt* flanked by two *loxP* sites into the first (vector A) and third (vector B) introns of *OsRac1* gene through homologous recombination (HR). DT-A encodings diphtheria toxin, functions as negative selection marker, was placed at both ends of vectors (Fig. 1B, Fig. 2B). We constructed different two vectors to introduce G59T mutation into *OsRac1* for the two reasons as follows. First, that was aimed to disperse a risk that modified *OsRac1* gene might become dysfunctional because of the change of its splicing pattern affected by the position of a *loxP* site left after *hpt*-Δ*En*/*Spm*

elimination. Thus, vector B with *loxP* left far away from the point mutation may reduce the risk. Second, it was predicted that the efficiency of mutant transfer from vector A is higher than that from vector B, due to nearer distance of the G56T mutation set in 5' homology region from *hpt* positive marker gene. Therefore, we constructed two vectors possessing different location of *hpt* to see the effect. In previous study, the transfer of several nucleotide changes set in the homology arms of GT vector into wild-type *Adh2* locus was analyzed, and the results showed frequency of transfer of nucleotide changes neighboring to the *hpt* gene was higher than those positioned away from the *hpt* (Jhozuka-Hisatomi et al., 2008).

3.1.2. True gene targeting lines are selected by positive-negative selection

Vector A and vector B were transformed into rice calli in independent GT experiments. The number of survived callus lines in *OsRac1* GT after positive-negative selection was 94 and 80 in vector A and vector B, respectively (Table 1). To select the true gene targeting events among the survived callus, PCR analysis with primer sets A5-F/A5-R and A3-F/A3-R corresponding to 5' and 3' junctions designed for vector A (Fig. 1B) or B5-F/B5-R and B3-F/B3-R primers designed for vector B (Fig. 2B) were carried out. 7 and 17 independent GT lines were obtained by transformation experiments with vector A and vector B, respectively (Table 1). All of them were sequenced around *OsRac1* exon 1 to confirm the point mutation. In total, 5 and 7 callus lines carried G56T point mutation in *OsRac1*-targeted were obtained with vector A and vector B, respectively. The rest did not show the transfer of G56T point mutation (Table 1). This was possibly generated by crossovers occurred on the right side of point mutation. As predicted, although vector B showed higher GT frequency, the frequency of substitution

was found lower than vector A. Probably, the homologous sequence on the right side of G56T in vector B was longer than that in vector A, thus more possible crossovers may occur in this region, leading to the integration of unit including *hpt* and two *loxP* sites, without G56T mutation. These callus lines carrying G56T mutation were named as A-1 to A-5 and B-1 to B-7 and propagated (Table 1).

3.1.3. *hpt* marker is eliminated by Cre-*loxP*-site specific recombination

5 and 7 callus lines obtained with vector A and vector B, respectively were subjected to elimination of *hpt*-Δ*En*/*Spm* fragment from *OsRac1* locus by ß-estradiol inducible XVE-Cre/*loxP* system in pBIMFN-*nptII* (Usuda et al., 2009, Zuo et al., 2001). Under ß-estradiol treatment, the inducible-Cre protein splices the fragment flanked by two *loxP* sites including *hpt* marker. Regenerated plants from shoots were grown individually on MS medium, and analyzed by PCR with primers ACL-F/ACL-R or BCL-F/BCL-R (Fig. 1B, Fig 2B) to select the desired transgenic carrying G56T substitution in *OsRac1* without *hpt*-Δ*En*/*Spm* fragment. As predicted, 4.3 kb fragment of *hpt* marker was not detected in the callus lines treated by Cre; whereas negative control samples without Cre treatment presented *hpt* fragment (Fig. 3A line 5-8, Fig. 4A line 5-8), indicating that *hpt* was removed completely from *OsRac1* locus.

3.1.4. *CA-gOsRac1* transgenic plants in the first generation are heterozygous

Since gene targeting plants generated by the same procedure with *OsRac1* in our previous studies followed the same heterozygous pattern, we genotyped *OsRac1* GT plants in the first generation by PCR with the same primer set applied to confirm the disappearance of *hpt* positive marker after Cre treatment: ACL-F/ACL-R and BCL-

F/BCL-R (Fig. 1C, Fig. 2C). As a result, gene targeting OsRac1 plants in the first generation (T0) showed both 0.54- and 0.59 kb fragments corresponding to wild type and mutant alleles, respectively. This result indicated that T0 *CA-gOsRac1* plants were heterozygous (Fig. 3A, Fig. 4A).

To confirm the genome structure of these plants, the *OsRac1* locus containing anticipated 5' and 3' recombination junction were amplified by PCR. The DNA fragments amplified from each plant were analyzed by direct sequencing. In addition, the 5' side fragments were cloned for the detailed analysis of G56T nucleotide substitution and its neighbor sequence including *loxP* site left in the first or third intron. As a result, 2 plants transformed with vector A and 2 plants transformed with vector B were confirmed as objective transgenic plants that had no errors and artificial sequences except for the G56T nucleotide substitution (Fig. 3B, Fig. 4B), and the 50 bp of insertion comprised of single *loxP* with restriction enzyme sites at intron1 and intron3, depend on the vector used. 4 plants (T_0) were grown up in greenhouse to harvest their seeds.

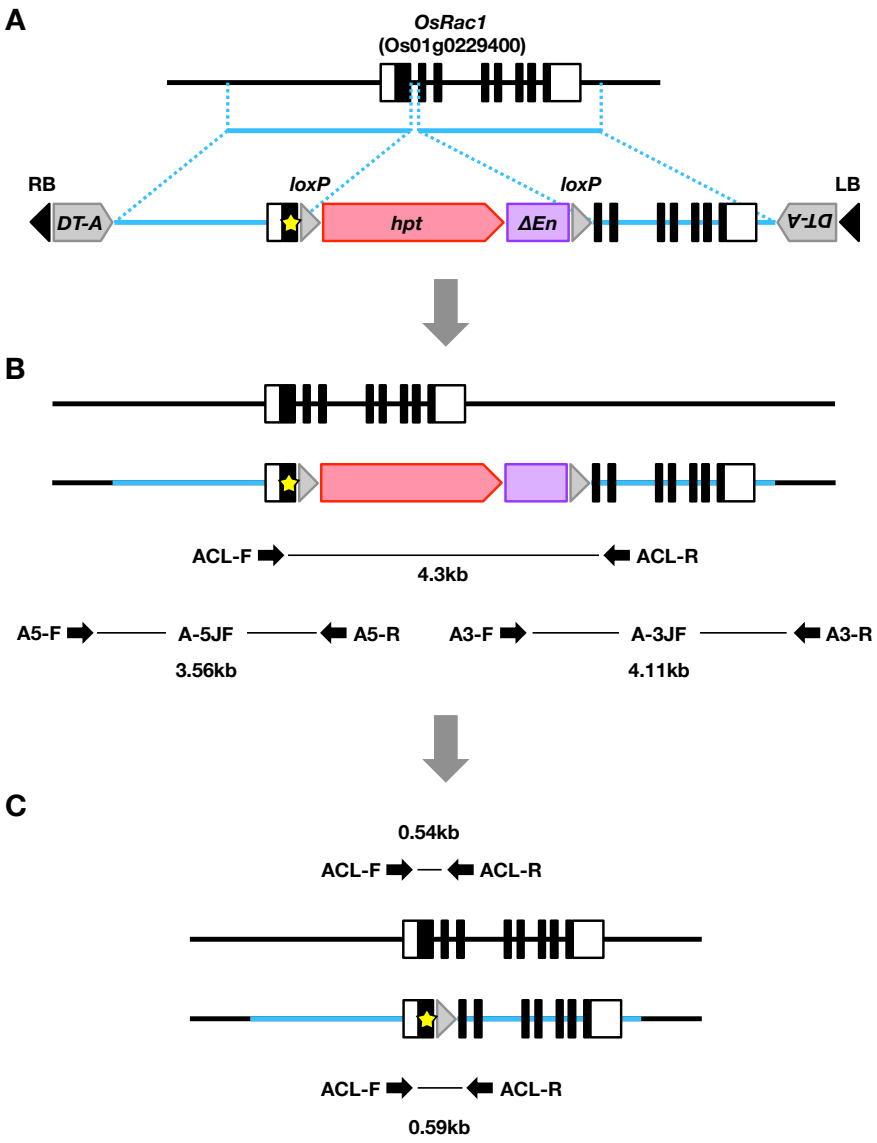


Figure 1. A strategy for modifying *OsRac1* by GT with vector A and subsequent Cre-*loxP* site-specific recombination.

(A) Schematic diagrams of *OsRac1* gene and T-DNA region of the targeting vector, Vector A. The black and white boxes indicate exons and UTRs, respectively. The light blue bars represent the sequence corresponding to the homologous regions carried by vector A. The yellow star in 5'-homologous region indicates the G56T nucleotide substitution that would be introduced to exon1 of endogenous *OsRac1* gene in Kinmaze genome. RB, right border; *DT-A*, *diphtheria toxin A* fragment gene for negative selection; *LoxP*, *LoxP* sites derived from bacteriophage P1; *hpt*, *hygromycin phosphotransferase* gene for positive selection; Δ*En*, functional transcriptional stop sequence from *En*/*Spm* transposon; LB, left border.

(B) Structure of the resultant *OsRac1*. Lines marked A-5JF and A-3JF indicate the 5' and 3' junction fragments generated by homologous recombination. Flanking black arrows marked by A5-F/A5-R and A3-F/A3-R represent primers used for PCR screening of targeted callus lines (Table 1). Amplification with primer set ACL-F/ACL-R generates a 4.3kb PCR fragment containing the *hpt*-Δ*En* region of *OsRac1* intron1.

(C) Structure of the modified *OsRac1* locus after Cre-*loxP* site-specific recombination.

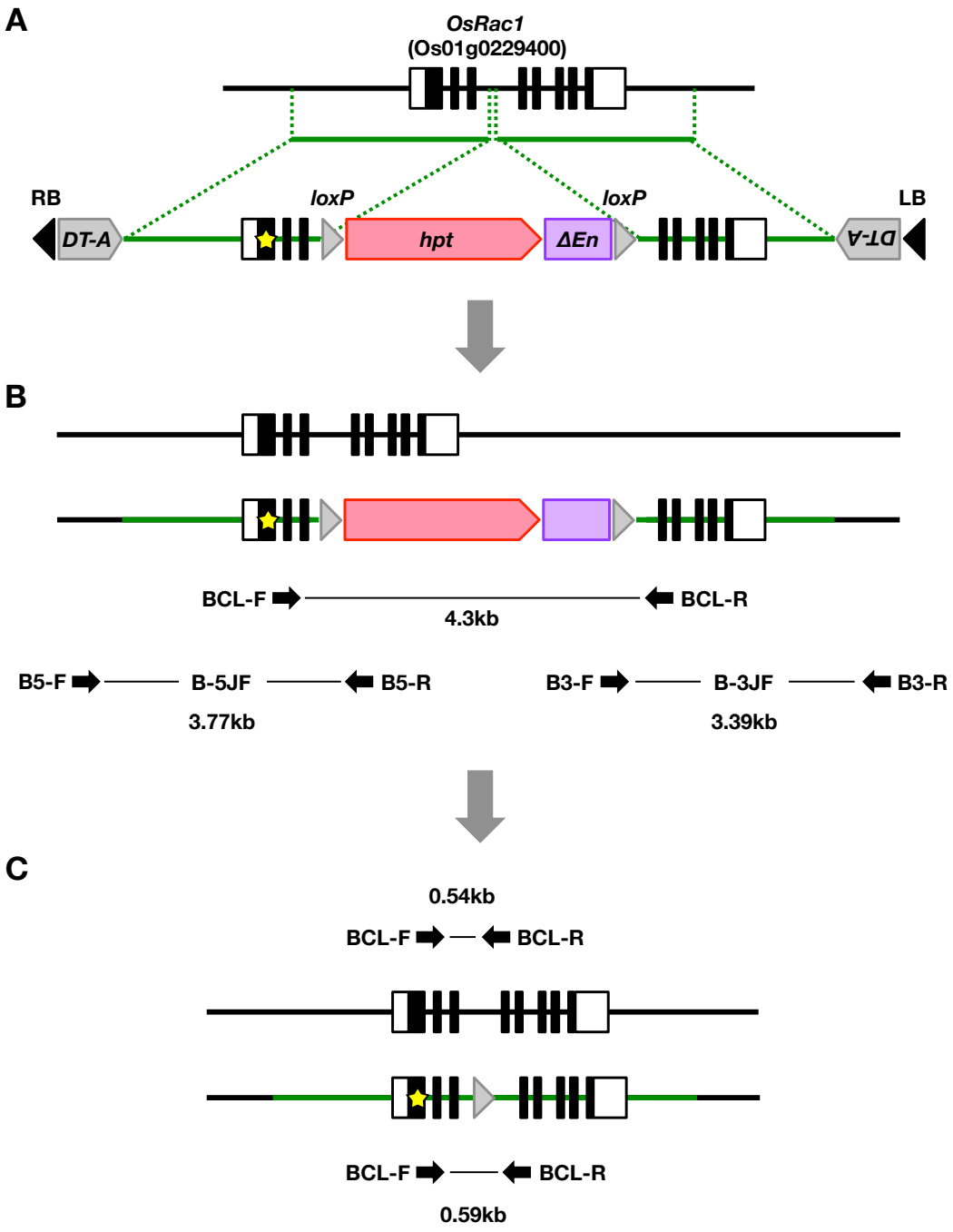


Figure 2. A strategy for modifying *OsRac1* by GT with vector B and subsequent Cre-*loxP* site-specific recombination.

(A) Schematic diagrams of *OsRac1* gene and T-DNA region of the targeting vector, vectorB. The black and white boxes are as in Fig.1A. The green bars represent the sequence corresponding to the homologous regions carried by vector B.

(B) Structure of the resultant *OsRac1*. Lines and black arrows are as in Fig.1B and Table 1. Amplification with primer set BCL-F/BCL-R generates a 4.3kb PCR fragment containing the *hpt*-Δ*En* region of *OsRac1* intron3.

(C) Structure of the modified *OsRac1* locus after Cre-*loxP* site-specific recombination

Table 1: Targeting frequency of *OsRac1*

Targeted gene	HM length on vector		Number of Exp. Seed number	PN selected Calli	TGT (5'+3')	Frequency TGT/PN (%)	Number of G to T substitution	Frequency of substitution (%)
	RB5'	LB3'						
OsRac1 (by pOsRac1-A)	3	3	2590	94	7	7.4	5	71
OsRac1(by pOsrac1-B)	3	3	2609	80	17	21.3	7	41

HM: Homologous sequence, PN: positive-negative selection, TGT: true gene targeting

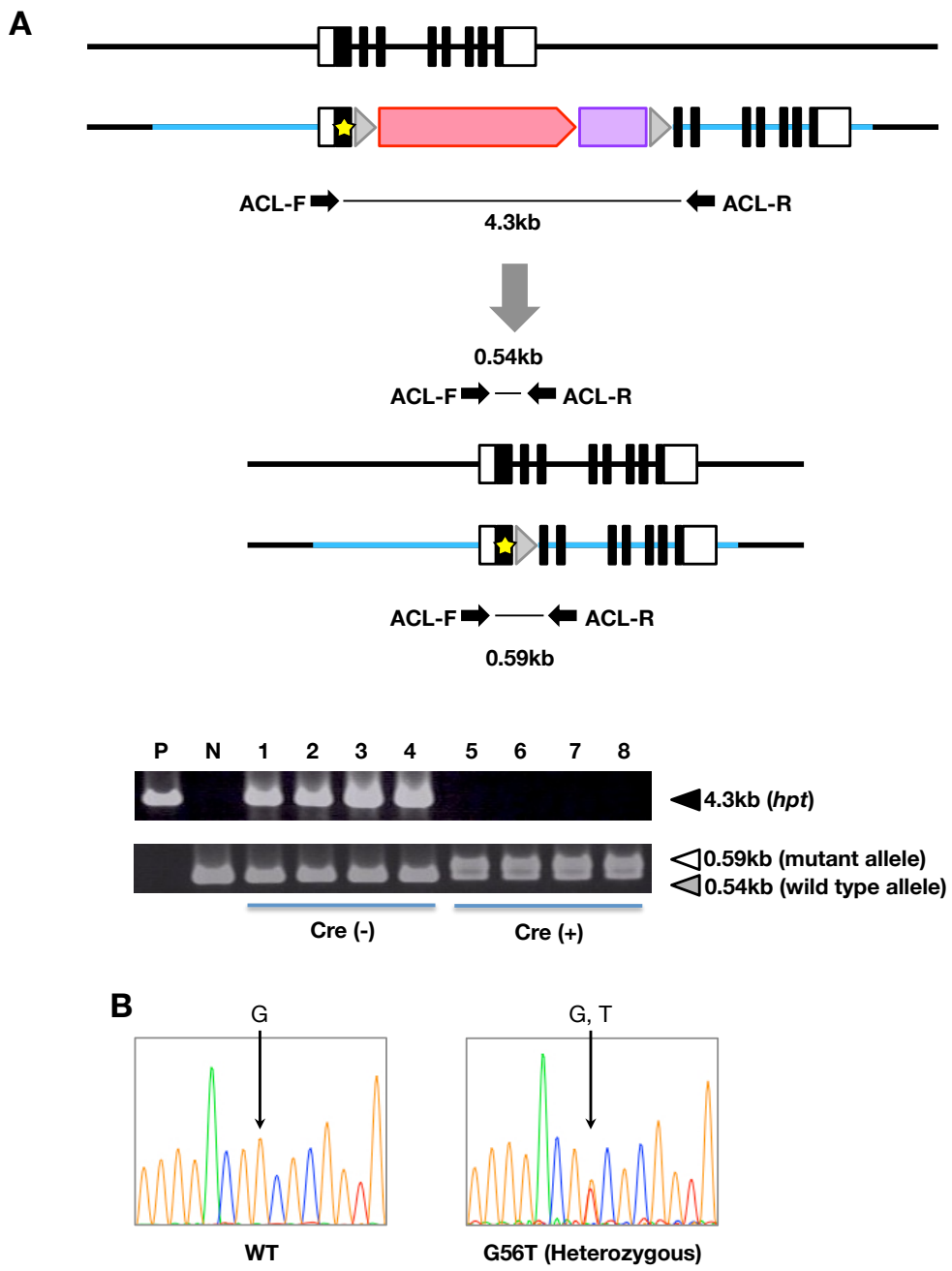


Fig. 3. PCR and DNA sequencing analysis of OsRac1-G19V mutants generated by GT with vector A.

(A) PCR screening for GT plants without *hpt*-Δ*En* fragment and genotyping T0 plants using primer sets ACL-F/ACL-R (Fig. 1B and 1C;); P, control plasmid containing A-5JF/A-3JF (Fig. 1B); N, non-transformed Kinmaze; Lane 1-4, T_0 GT plants harboring *hpt*-Δ*En* fragment in *OsRac1* intron1; Lane 5-8, T_0 GT plants carrying a *loxP* "footprint" indicating successful elimination of *hpt*-Δ*En* fragment by Cre-*loxP* site-specific recombination. Black and white arrowheads indicate the insertion and excision of *hpt*-Δ*En* fragment in *OsRac1* intron1, respectively. Gray arrowhead indicates the wild type *OsRac1* gene without a *loxP* site.

(B) DNA sequence of the G56T nucleotide substitution in *OsRac1* exon1 of transgenic plant.

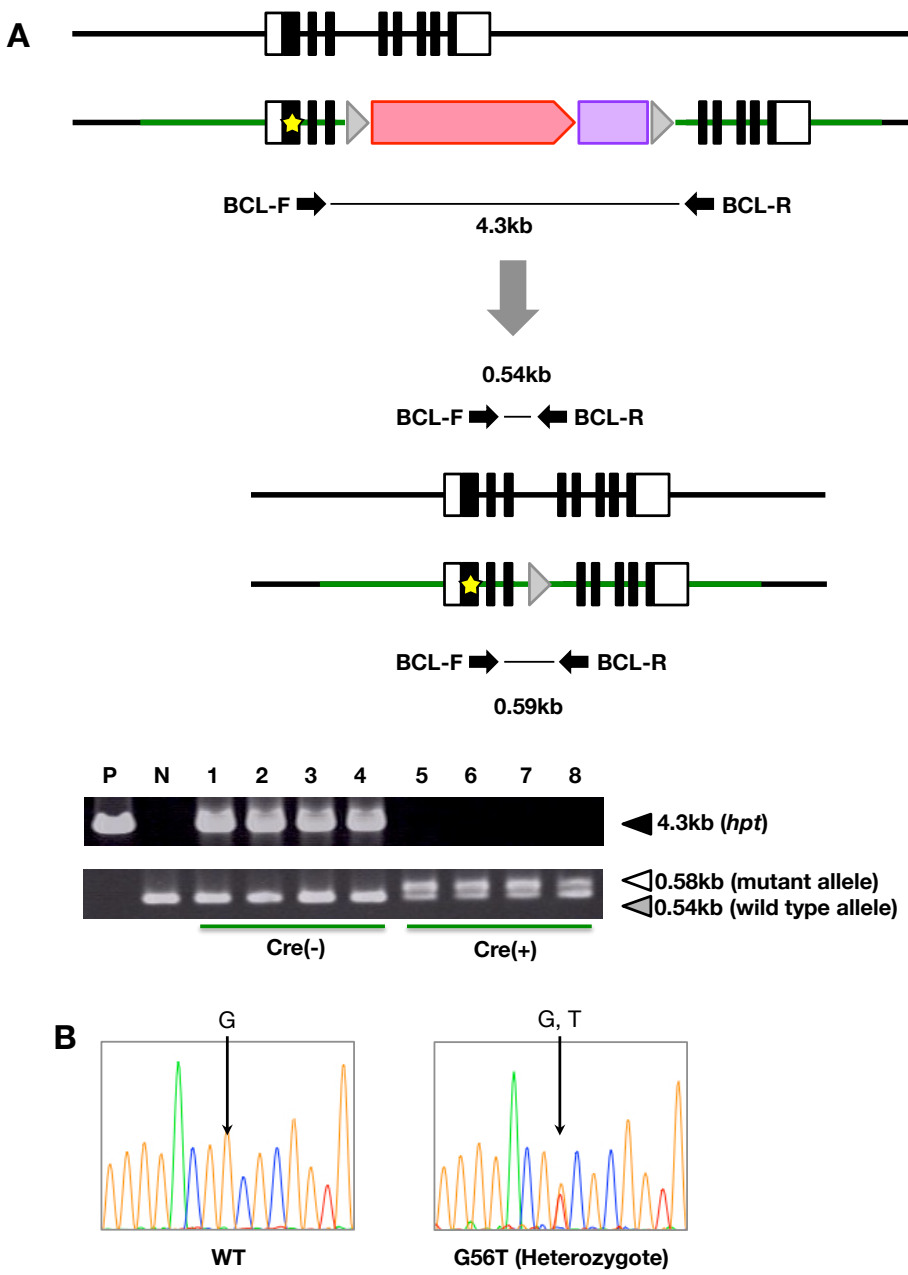


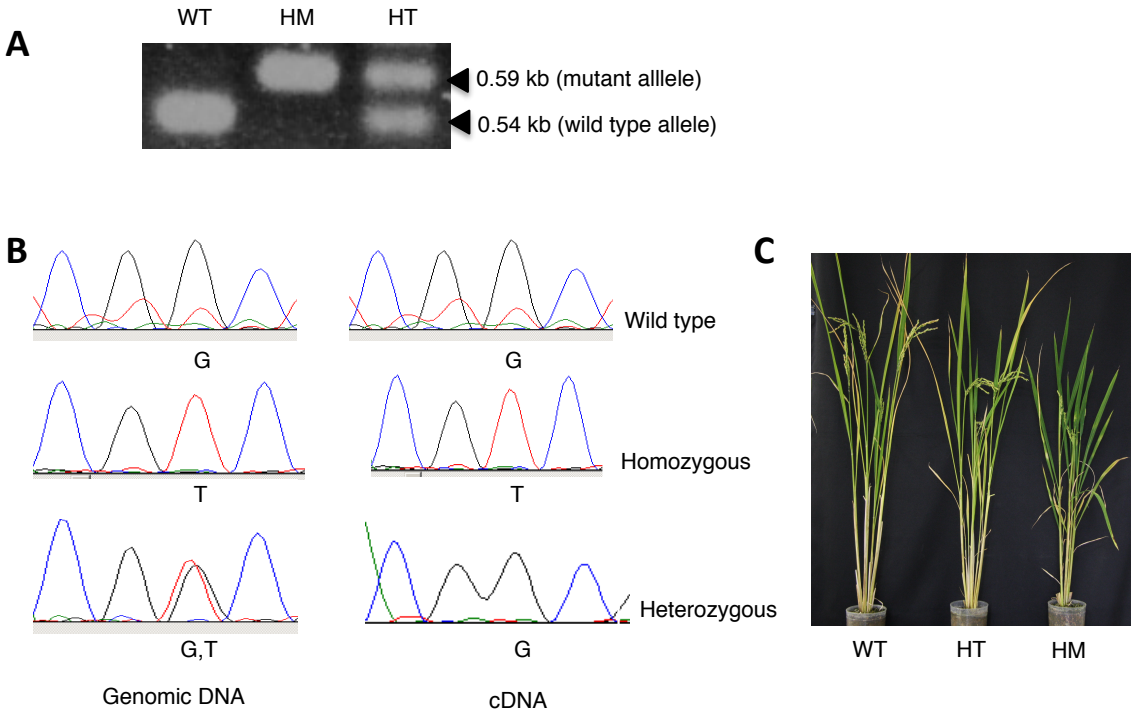
Fig. 4. PCR and DNA sequencing analysis of OsRac1-G19V mutants generated by GT with vector B.

(A) PCR screening for GT plants without *hpt*-Δ*En* fragment and genotyping T0 plants using primer sets BCL-F/BCL-R (Fig. 2B and 2C; Table 1); P, control plasmid containing B-5JF/B-3JF (Fig. 2B); N, non-transformed Kinmaze; Lane 1-4, T_0 GT plants harboring *hpt*-Δ*En* fragment in *OsRac1* intron3; Lane 5-8, T_0 GT plants carrying a *loxP* "footprint" indicating successful elimination of *hpt*-Δ*En* fragment by Cre-*loxP* site-specific recombination. Black and white arrowheads indicate the insertion and excision of *hpt*-Δ*En* fragment in *OsRac1* intron 3, respectively. Gray arrowhead indicates the wild type *OsRac1* gene without a *loxP* site.

(B) DNA sequence of the G56T nucleotide substitution in *OsRac1* exon1 of transgenic plant.

3.2. G56T nucleotide substitution is transmitted stably to the next generation and expressed at the mRNA level.

Unfortunately, two plants targeted by vector B did not produce seeds for the next generation for unknown reasons; therefore they could not be used for the subsequent experiments. All the following experiments were performed on the plants transformed with vector A. Since transgenic plants in the first generation were heterozygous, the mutant allele should be segregated in T1 generation. To identify genotypes of the T1 progeny, PCR analysis combined with direct sequencing of the genomic *OsRac1* were performed. A 0.54 kb fragment corresponding to the wild type *OsRac1*, and the 0.59 kb fragment for the homozygous *OsRac1* with the *loxP* insertion were detected, while heterozygous plants carried both 0.54 and 0.59 kb fragments (Fig. 5A). Moreover, all the T1 plants were sequenced to ensure the presence of G56T nucleotide substitution. As expected, plants with wild type and homozygous G56T nucleotide substitution mutation harbored the wild type and nucleotide substitution mutation, respectively, whereas those with heterozygous showed both wild type and mutant peaks (Fig. 5B). T1 generation plants segregated 29 homozygous, 40 heterozygous, and 29 wild type plants (Fig. 5D). Homozygous plants were slightly dwarf and heterozygous plants were not distinguishable from the wild type plants (Fig. 5C). Taken together, G56T nucleotide substitution targeted by vector A was transmitted stably to the next generation. Point mutation was further confirmed at the mRNA level by directly sequencing *OsRac1* cDNA (Fig 5B) The sequence trace of the G56T mutant peak in a homozygous plant demonstrated that the mutated *OsRac1* was expressed at the mRNA level. However, the point mutation was not detected in the cDNA of heterozygous plants, probably due to the low expression level of mutant transcripts (see below).



	Number of plants		
Genotype	Plant 98	Plant 110	Total
Wild type	19	10	29
Heterozygous	24	16	40
Homozygous	16	13	29

Fig. 5. G56T nucleotide substitution was stably transmitted to the next generation and expressed at the mRNA level. (A) PCR analysis to identify the genotypes of T1 generation. Genotypes of homozygous (HM), heterozygous (HT) and wild type (WT) plants are shown with predicted bands. (B) Sequencing chromatogram of transgenic plants in T1 generation at genomic DNA extracted from leaf blade and cDNA synthesized from leaf sheath. G56T nucleotide substitution and wild type *OsRac1* were illustrated by base change and wild type peak, respectively in both genomic DNA and cDNA. Heterozygous plants showed overlapping peaks in genomic DNA, but only wild type peak was detected in cDNA. (C) Phenotypes of *CA-gOsRac1* gene targeting plants in the second generation. (D) Segregation analysis of transgenic plants in the second generation.

3.3. The mutated *CA-gOsRac1* expression is suppressed at the mRNA level.

Since we could not detect the sequence trace of G56T nucleotide substitution in the cDNA of heterozygous plant, expression levels of *OsRac1* were examined in various tissues by quantitative reverse transcription-PCR (qRT-PCR) (Fig. 6). The low level of *CA-gOsRac1* expression in the homozygous plants was found in all tested tissues. In addition, *CA-gOsRac1* was almost fully suppressed in leaf blade and cell cultures (Fig. 6A, 6B), but partially suppressed in leaf sheath, root and panicle (Fig. 6C-E). These results suggested that the expression of *CA-gOsRac1* was repressed at the mRNA level and in a tissue-specific manner. At present, the reasons for low expression of mutant transcripts remain elusive.

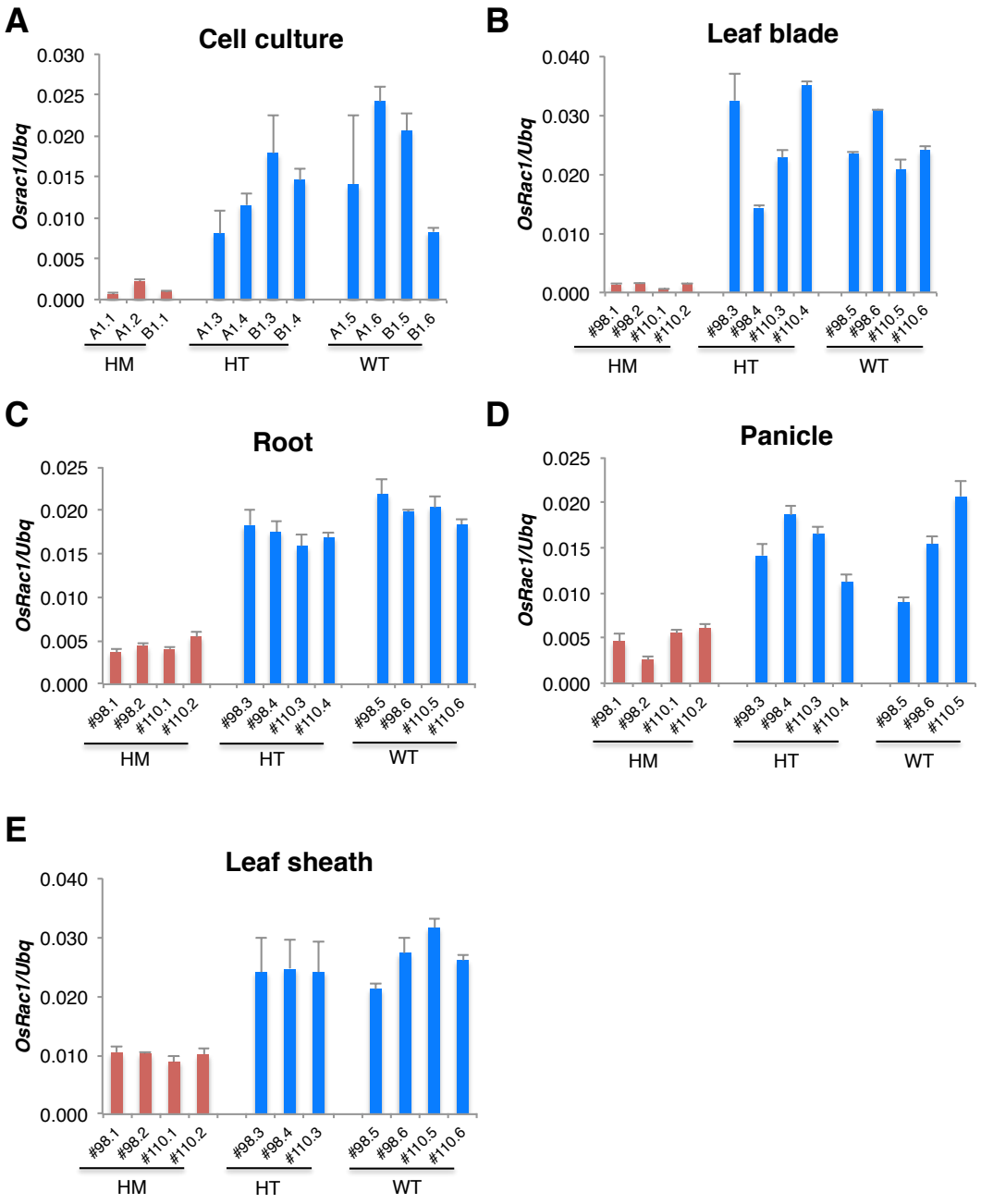


Fig. 6. Expression of *CA-gOsRac1* in various tissues of homozygous transgenic rice plants. Total mRNA was extracted from various tissues by Trizol method (Invitrogen). *OsRac1* cDNA was synthesized from 1μg mRNA and monitored the expression of *OsRac1* by qRT-PCR. *Ubq* was used as an internal control. Bars represent ± SD, n=3

3.4. *CA-gOsRac1* enhances chitin-triggered immune response and induces hyper response (HR) cell death.

OsRac1 is involved in the induction of defense related genes such as *PAL1* and *PBZ1* in chitin pathway (Thao et al., 2007, Chen et al., 2010, Akamatsu et al., 2013). To examine whether homozygous *CA-gOsRac1* exhibit enhanced chitin induced immune response, *CA-gOsRac1* cell cultures were treated with chitin and expression of *PAL1* and *PBZ1* were examined at various times after the treatment. Compared to the wild type, homozygous *CA-gOsRac1* showed strong induction of *PAL1* and *PBZ1* at 1 hour and 9 hour upon chitin treatment, respectively. In heterozygous plants, however *PAL1* was highly activated, but *PBZ1* was not (Fig. 7A). Probably, *PAL1* is known to be strongly and quickly induced not only by pathogens but also by other environmental stress (Minami et al., 1996), hence one mutant transcript was sufficient for its enhancement. In contrast, accumulation of *PBZ1* appears to be specific to blast fungus infection and chitin treatment (Nakashita et al., 2001), thus two *OsRac1* mutant transcripts were required to sharply induce its expression. These results indicated that although expression of *CA-gOsRac1* was very low in cell cultures, its expression level was sufficient to enhance chitin-triggered immune responses compared to the wild type.

Next, we examined the response of homozygous *CA-gOsRac1* plants to infection by blast fungus *Magnaporthe oryzae (M. oryzae)* compatible race 007. Since the expression of homozygous *CA-gOsRac1* was not strongly suppressed in leaf sheath among the tested tissues, it was selected for infection by using intact injection method (Koga et al., 2004). Three days after inoculation of spores into leaf sheath, we observed the appearance of granules with strong auto-fluorescence and strong trypan blue staining (Fig. 7B), a histochemical indicator of irreversible membrane damage or cell death (Yin

et al., 2000) in both homozygous and heterozygous plants. These results suggested that one mutant transcript was sufficient to induce hypersensitive response-like cell death by infection with a compatible race of blast fungus. In contrast, in wild type plants, fungal hyphae invaded to neighboring leaf cells and HR-like cell death was not detected (Fig. 7B). Together, these results demonstrated that *CA-gOsRac1* plants generated by gene targeting showed enhanced stronger immune response such as enhanced defense-related genes activation by the chitin elicitor and HR-like cell death after infection with a compatible rice blast fungus, which was not detected in the wild type plants.

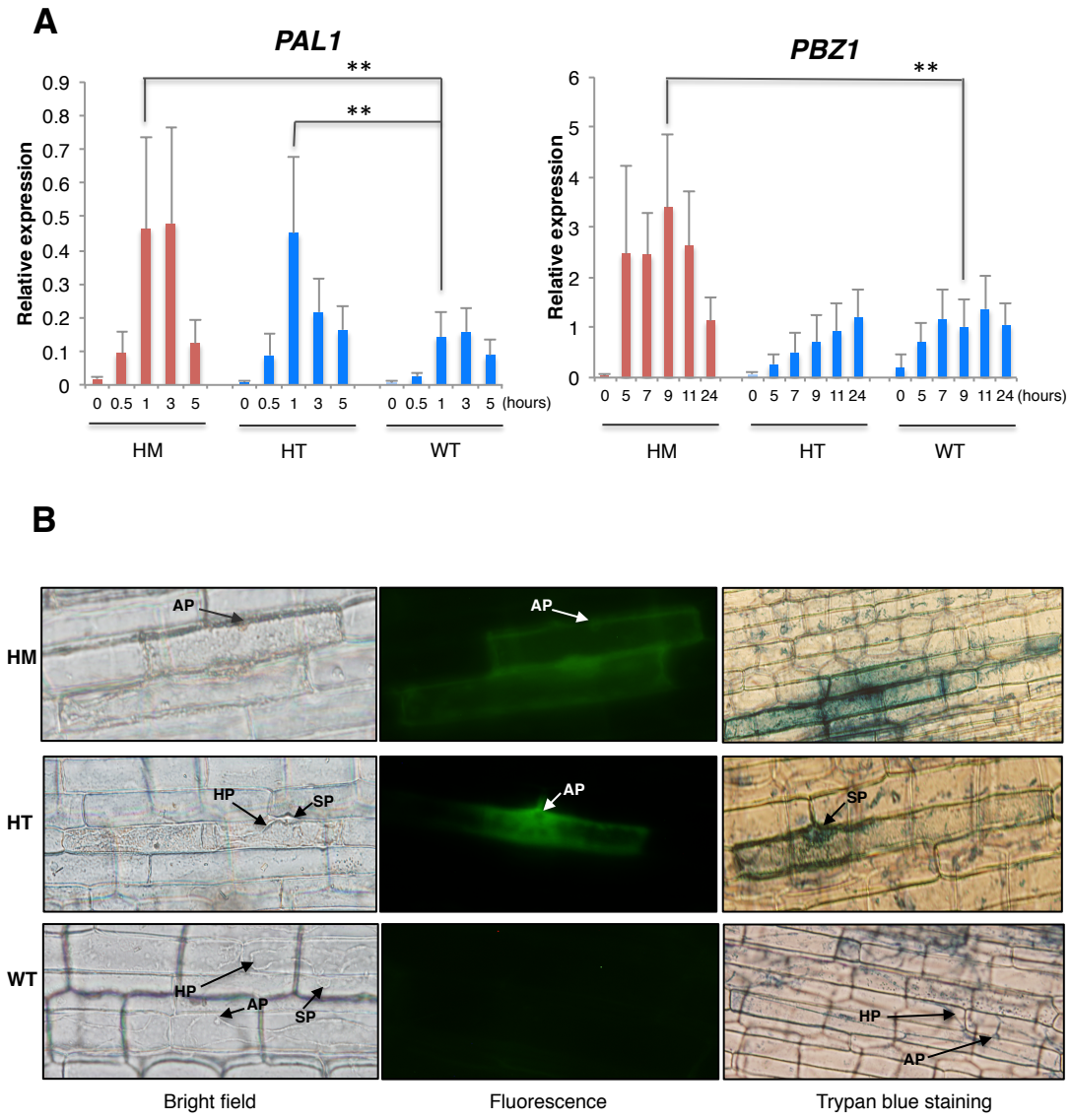


Fig. 7. Homozygous *CA-gOsRac1* plants activate expression of pathogen related genes and induces HR-like cell death. (A) Expression of *PAL1* and *PBZ1* upon chitin treatment. Cell cultures were treated with 20 μg/ml chitin. Cells were collected at indicated time points and the expression of *PAL1* and *PBZ1* was monitored by q-PCR. *Ubq* was used as an internal control. Bars represent ± SD, n=3. Double asterisks illustrate a significant difference between indicated samples (student's T-test; $P < 0.05$). (B) Induction of HR-like cell death in homozygous plants infected with *M. oryzae* compatible race 007. Ap: Appresorium generated from fungus spore; HP: Hyphae; Sp: Spore.

3.5. *CA-gOsRac1* activates defense-related genes induced by *M. oryzae* and *Xanthomonas oryzae pv.oryzae* (*Xoo*) in the absence of infection

To examine the expression profile of homozygous *CA-gOsRac1*, microarray analysis was performed with mRNAs extracted from non-infected leaf sheath and the results were compared with rice gene profiles reported to be induced by *M. oryzae* and *Xoo* infection (Kottapalli et al., 2007, Mosquera et al., 2009, and Grewal et al., 2012). Analysis with Agilent Rice gene microarrays was conducted to examine transcripts accumulation of more than 2 fold different from wild type plants with P value < 0.05. Among 115 genes significantly induced in wild type plants by both *M. oryzae* and *Xoo* infection, we found 25 of them enhanced in homozygous *CA-gOsRac1* plant, the rest 90 transcripts showed either down-regulation or unchanged in *CA-gOsRac1*. Thus, *CA-gOsRac1* seems to contribute to 21.74% of transcripts enhanced by *M. oryzae* and *Xoo* infection. 25 transripts were grouped into distinct clusters: transcription factor, kinase, defense related, chitinase, peroxidase and others (Table 3). To validate the fold induction from microarray assay, 12 randomly selected transcripts were quantified by q-PCR. All of them showed the enhanced expression in the homozygous plants compared to the wild type plants, validating the results obtained in the microarray assay (Fig. 8). The induced transcripts are as follows.

Transcription factor: Plant host cells recognize the pathogen through plasma membrane and stimulate diverse signaling pathways including MAPK cascade, Ca^{2+} signaling, ionic fluxes. These signals are transduced into nucleus, resulting in up regulation of transcription factors. The transcription factors, in turns regulate the expression of defense related genes, hormone and growth related genes (Grewal et al., 2012). In present study, three members of WRKY transcription factors family were markedly induced in the

homozygous *CA-gOsRac1* plants. According to Du et al (2000), WRKY transcription factors bind specifically to W box in the promoter region of pathogen related and SA genes. WRKYs also have a self-regulatory mechanism among their super-family (Dong et al., 2003). Three members of the WRKY transcription factor family were markedly induced in the homozygous *CA-gOsRac1*. Expression of WRKY69, a well documented member of this family involved in the regulation of disease resistance through MAPK cascade (Rushton et al., 2010) was increased. Expression of OsNac4 transcription factor, a positive regulator of HR cell death by regulating OsHSP90 and IREN (Kaneda et al., 2009) was also higher in homozygous *CA-gOsRac1*. Homozygous *CA-gOsRac1* plants significantly induced OsMYB4 expression, whose ectopic expression in *Arabidopsis* stimulates a set of genes involved in pathogen resistance such as AtERF6, EREBP4, WRKY6 (Vanini et al., 2006). In addition, OsMYB4 induces genes encoding enzymes in the phenylpropanoid pathway important for plant immunity (Vanili et al., 2006). Furthermore, ten-fold increase in expression was measured for ethylene responsive element binding factor 5 (ERF5) that regulates genes in chitin-triggered responses including ERF6, ERF8 and SCl13 as well as MPK3 and MPK6. There are conflicting reports about the functions of ERF5/ERF6 in plant defense responses in recent publications. Son et al (2012) reported that ERF5/ERF6 negatively regulate plant defense against the fungal pathogen *Alternaria brassicicola* and positively regulate plant defense against the bacterial pathogen *pseudomonas syringae pv tomato* DC3000. In contrast, according to Moffat et al (2012), ERF5/ERF6 play a positive role in plant resistance against *B. cinerea*, a necrotrophic fungal pathogen with the same life style as *A. brassicicola*, and a negative role in resistance to *P. syringae pv tomato* DC3000.

Protein kinases are found to play a critical role in signaling transduction concerned with disease resistance (Grewal et al., 2012). Among this class, mitogen activated protein kinase kinase kinase 3 (MAPKKK3) (MAP kinase kinase kinase alpha) expression increased more than 5-fold in homozygous *CA-gOsRac1* plants. Similarly, expression of *Receptor-like protein kinase 5 precursor (CRK5)* increased more than 3-fold. CRK5 overexpression significantly enhances disease resistance in Arabidopsis (Chen et al., 2003).

Other genes: Expression of genes for chitinases and peroxidases involved in immune responses was greater in homozygous *CA-gOsRac1* plants than wild type. Genes for lipoxygenases that are key enzymes for lipid metabolism and jasmonic acid (JA) biosynthesis also showed enhanced expression. LOX2 is required for wound-induced JA accumulation and is involved in the early defense response to pathogens (Creelman et al., 1997). *OsChi3b* encodes a chitinase that has anti-fugal activity by its ability to hydrolyze N-acetylglucosamine polymers (Punja et al., 1993) and was induced 12-fold in the homozygous plants. In addition, enhanced expression of peroxidases, important enzymes for plant defense against pathogenic microorganisms was found. In the absence of pathogen infection, the results indicated that homozygous *CA-gOsRac1* plants constitutively induced a larger number of genes involved in immune responses. These results support the notion that OsRac1 plays a key role in rice immunity.

Table 3: List of genes induced in homozygous *CA-gOsRac1* plants which are previously shown to be induced by *M. oryzae* and *Xoo* infections, P<0.05

	Gene ID	Fold change by *M. oryzae* (a)	Fold change by Xoo (b)			Fold change in HM (Data 1)	Fold change in HM (Data 2)	Annotation
		72 h	1 h	6 h	120 h			
Transcription factors	AK107199	3		3.9	3.1	3.3	14.9	WRKY transcription factor 24
	AK106282	19		2.0	2.8	5.5	26.6	WRKY transcription factor 28
	AK111606		3.8	4.9	6.5	3.0	5.0	WRKY transcription factor 69
	AK 073848	64	2.1	1.8		3.4	1.5	Similar to OsNAC4
	AK112056			5.5	4.6	30.4	1.5	Similar to Y19 protein (MYB4)
	AK073812		2.6	1.7	2.3	10.4	18.4	Similar to ERF5
Kinases	AK059839			7.6	2.0	2.2	15.4	Zinc finger, C2H2 type domain containing protein
	AK107566	12				3.6	3.0	LRR, N-terminal domain containing protein
	AK100082	5				4.1	3.7	CRK5 5 precusor
	AK068504		2.5			3.8	3.4	Similar to receptor-like protein kinase
Defense related genes	AK068725		2.7			5.1	8.1	MAPKKK3
	AK066825	14	10.2			4.2	6.1	Similar to Lipoxygenase
	AK071546	68				13.4	15.0	Similar to cytochrome P450 CYP99A1
Chitinases	AK104472		6.7			5.5	2.9	Similar to Flavonoid 3-monoxygenase
	AK059767	20				14.6	1.6	Similar to Chitinase 1 precursor
	AK100973	9	8.1	4.6	2.8	58.3	12.9	OsChi3b precursor
Peroxidases	AK102505	7				25.4	3.2	Similar to class III chitinase homologue
	AK104277	13				9.3	2.9	Similar to peroxidase BP-1 precursor
	AK067667	5				4.9	2.7	Peroxidase (1.11.1.7)
	AK073202	3				3.7	15.5	Similar to peroxidase
Others	AK063042	58				7.0	6.1	DUF581 family protein
	AK061337		10.8	2.0	1.9	6.9	2.5	Flavanone 3-hydroxylase-like protein
	AK063639			2.1	6.8	18.9	2.4	Cupredoxin domain containing protein
	AK062449			1.9	2.2	5.5	22.8	RIR1 protein precursor
	AK100411		3.4			10.5	3.4	Similar to Ammonium transporter

(a): Data are from Mosquera et al., 2009; (b): Data are from Grewal et al., 2012 and Kottapalli et al., 2007

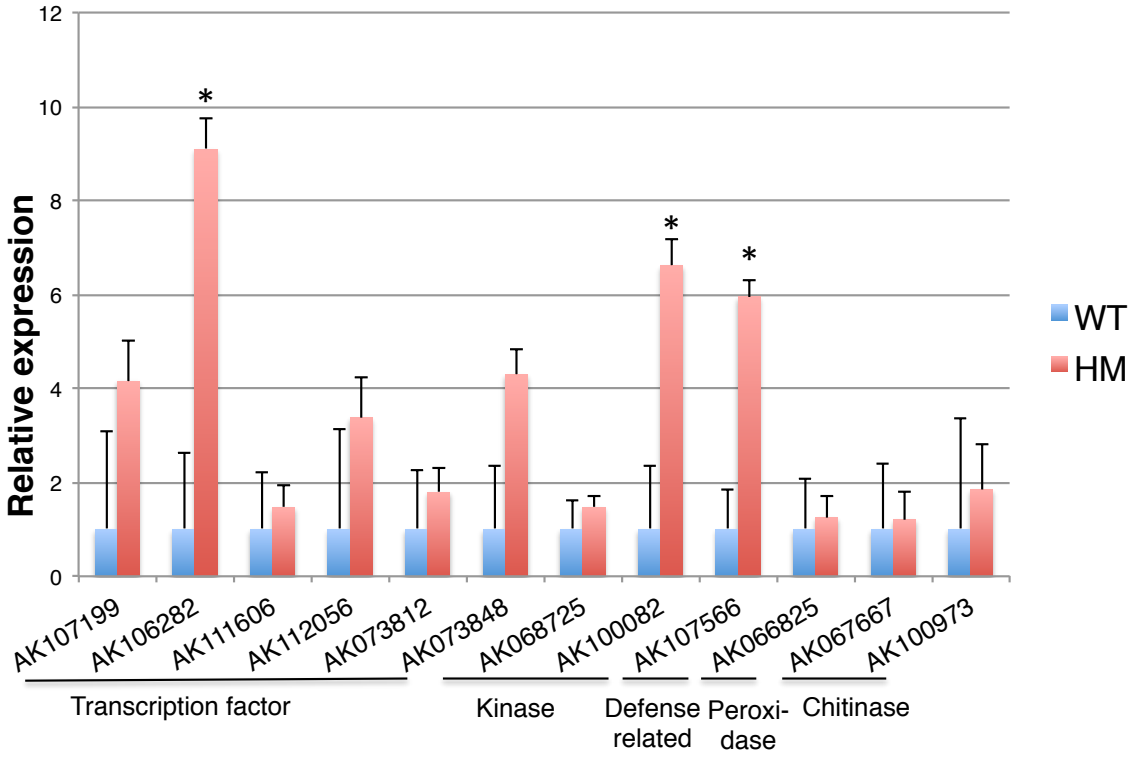


Fig. 8. Validation of the microarray data by qPCR. Twelve transcripts, which showed more than 2-fold up-regulation, were randomly selected from each cluster and quantified by qPCR. The same mRNAs used for the microarray and further four mRNAs extracted from leaf sheath of homozygous and wild type plants were used in this experiment. *Ubq* was used as an internal control. Bars indicate ± SD, n=6. Asterisks indicate the significant different between specified samples, student's t-test, $P<0.05$

3.6. *CA-gOsRac1* rice plants are not able to enhance rice blast resistance

Previous studies showed that *CA-OsRac1* plants enhance disease resistance to both blast fungus *M. oryzae* and bacterial blight *Xoo* (Ono et al., 2001). To examine the disease resistance of *CA-gOsRac1* plants, infection assay was carried out to introduce both compatible race 007 and incompatible race 031 of rice blast fungus *M. oryzae* into leaf blade of *CA-gOsRac1* (Chen et al., 2010a). The lesion length and infection ratio (*M. oryzae/Ubq* x 100) monitored by real-time PCR were applied to evaluate the disease resistance of *CA-gOsRac1* plants (Fig. 9). As a result, wild type, heterozygous and homozygous *CA-gOsRac1* did not show significant difference in lesion length as well as infection ratio, suggesting that *CA-gOsRac1* homozygous plants were insufficient to increase disease resistance. Probably, the expression of *CA-gOsRac1* mutant was too low to confer enhanced disease resistance.

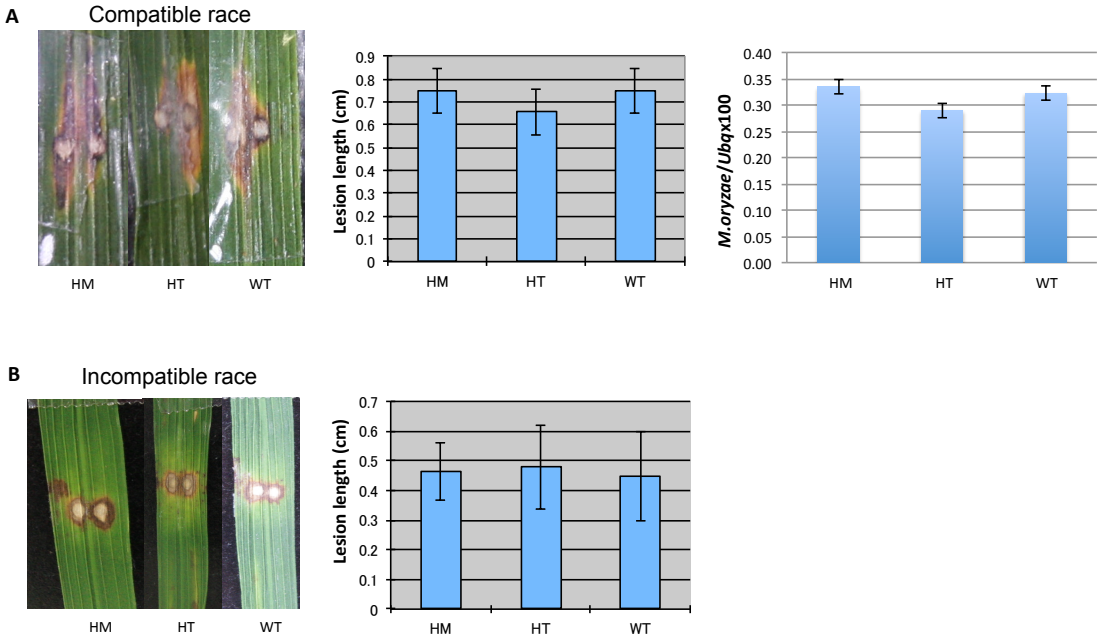


Fig. 9. Infection assays of *CA-gOsRac1* rice plants with compatible race (007) (A) and incompatible race 031 (B). Pictures (left) show typical phenotype of infected leaf blade. Lesion lengths and infection ratio measured by real-time PCR are shown with ± SD, n=24 (student's t-test, P>0.05).

3.7. Reasons for low expression of *CA-gOsRac1* at the mRNA level

In examining the expression of *OsRac1* at the mRNA level, unfortunately we found the suppression of mutant transcripts in homozygous *CA-gOsRac1* from all tested tissues (Fig. 6). It is possible that potential detrimental effect of the constitutively active G19V substitution may affect the expression of *OsRac1*. To examine this possibility we compared the expression levels of *OsRac1* in homozygous plants containing active G56T substitution plus *loxP* sequence (type 1) (Fig. 10) generated by vector A and those with *loxP* alone (type 2) (Fig. 10) generated by vector B. Interestingly, the level of *OsRac1* expression in the homozygous *loxP* without point mutation was not suppressed (Fig. 10), suggesting that the phenotypic effect of the G56T nucleotide substitution may have caused the low *OsRac1* expression. However, because type 1 and type 2 plants used in this experiment possessing different location of *loxP* (Fig. 10), we could not rule out the possibility that different integration sites of *loxP* may have affected levels of *CA-OsRac1* expression.

To examine whether the low expression level of mutant transcripts was due to the possible interference of *loxP* on the splicing pattern of the mRNA *CA-gOsRac1*, Northern blotting was performed with the mRNA extracted from leaf sheath of wild type, heterozygous and homozygous plants (Fig. 11). As a result, no extra spliced forms of the mRNA *OsRac1* was detected in homozygous and heterozygous plants, suggesting that 50-bp *loxP* located in the first intron may not disturb the splicing pattern of the mRNA *CA-gOsRac1*.

To test a possibility that suppression of *OsRac1* expression was due to inactivation of *OsRac1* by DNA methylation, we analyzed the methylation status of 1 kb fragment in the promoter region of *OsRac1* from the transcription start point including the TATA box (Fig. 12) and a part of gene body surrounding *loxP* position (Fig. 13) by bisulfite sequencing. However,

no significant difference in the methylation levels of three cytosine patterns CG, CHG, CHH between wild type and the homozygous *CA-gOsRac1* plants was detected (Fig 12, Fig. 13), suggesting that inactivation of *OsRac1* promoter by DNA methylation was not likely to be the reason for the suppressed *OsRac1* expression.

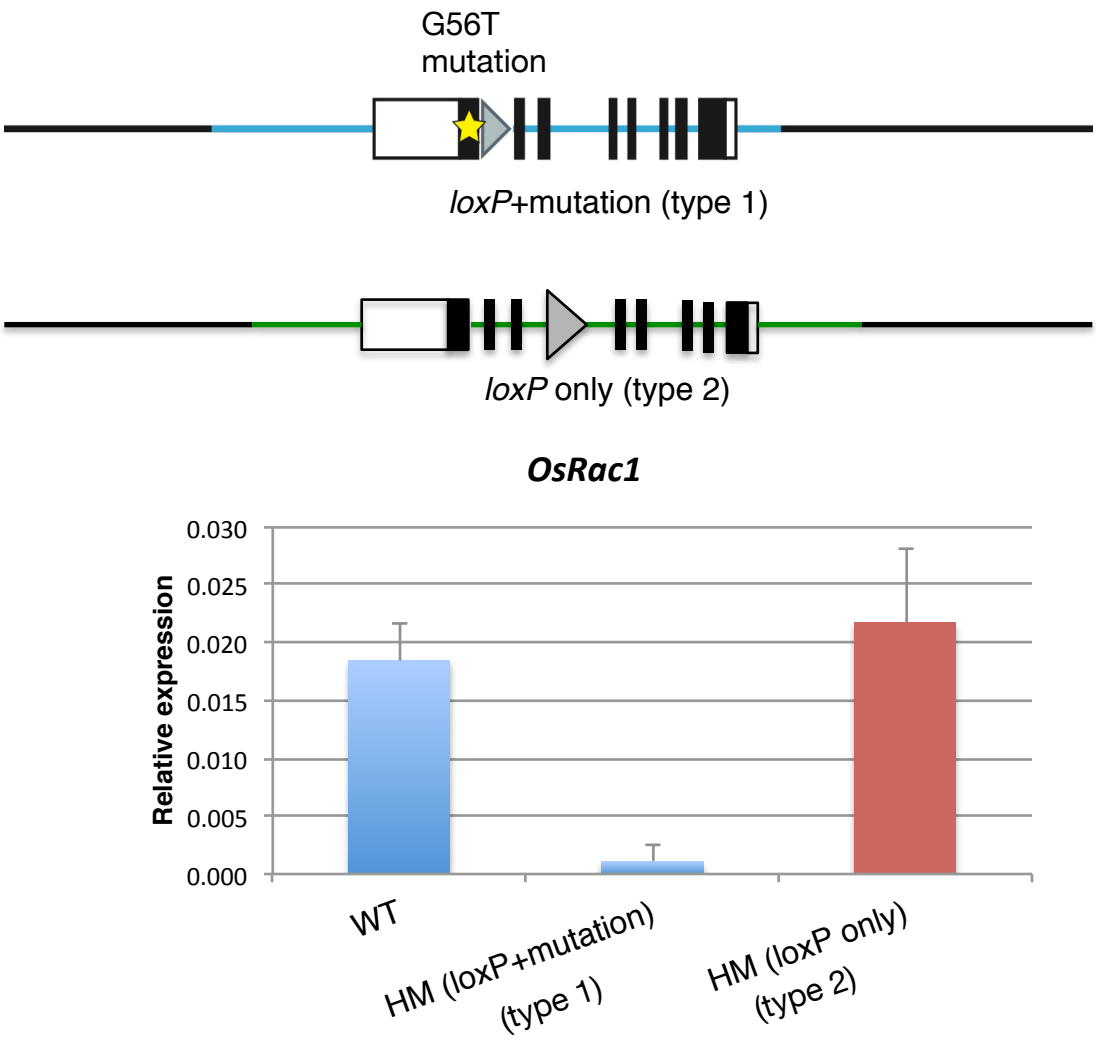


Fig. 10. Transgenic plants containing *loxP* without point mutation was not repressed at the mRNA level. q-PCR was performed to examine the expression level of *OsRac1* from the leaf blade of homozygous plants carrying the point mutation plus *loxP* generated by pOsRac1G19V-A and those possessing *loxP* alone generated by pOsRac1G19V-B. *Ubq* was used as an internal control. Bars represent ± SD, n=3

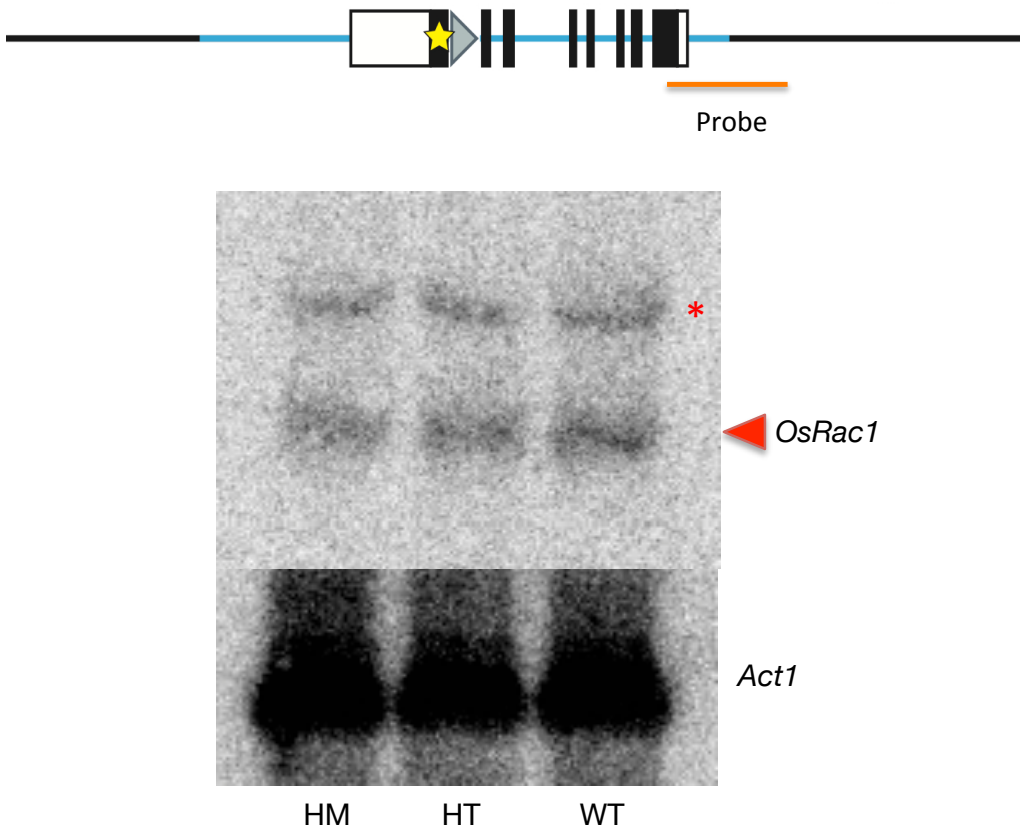


Fig. 11. No extra spliced form of *CA-gOsRac1* is detected.

Northern blotting was performed with the mRNA extracted from leaf sheath of homozygous (HM), heterozygous (HT) and wild type (WT) plants. The probe was designed to align with the region between the last exon and the 3'UTR region of the mRNA *OsRac1* (396 bp). *Actin1 (Act1)* gene was used as an internal control. * indicates the position of non-specific bands.

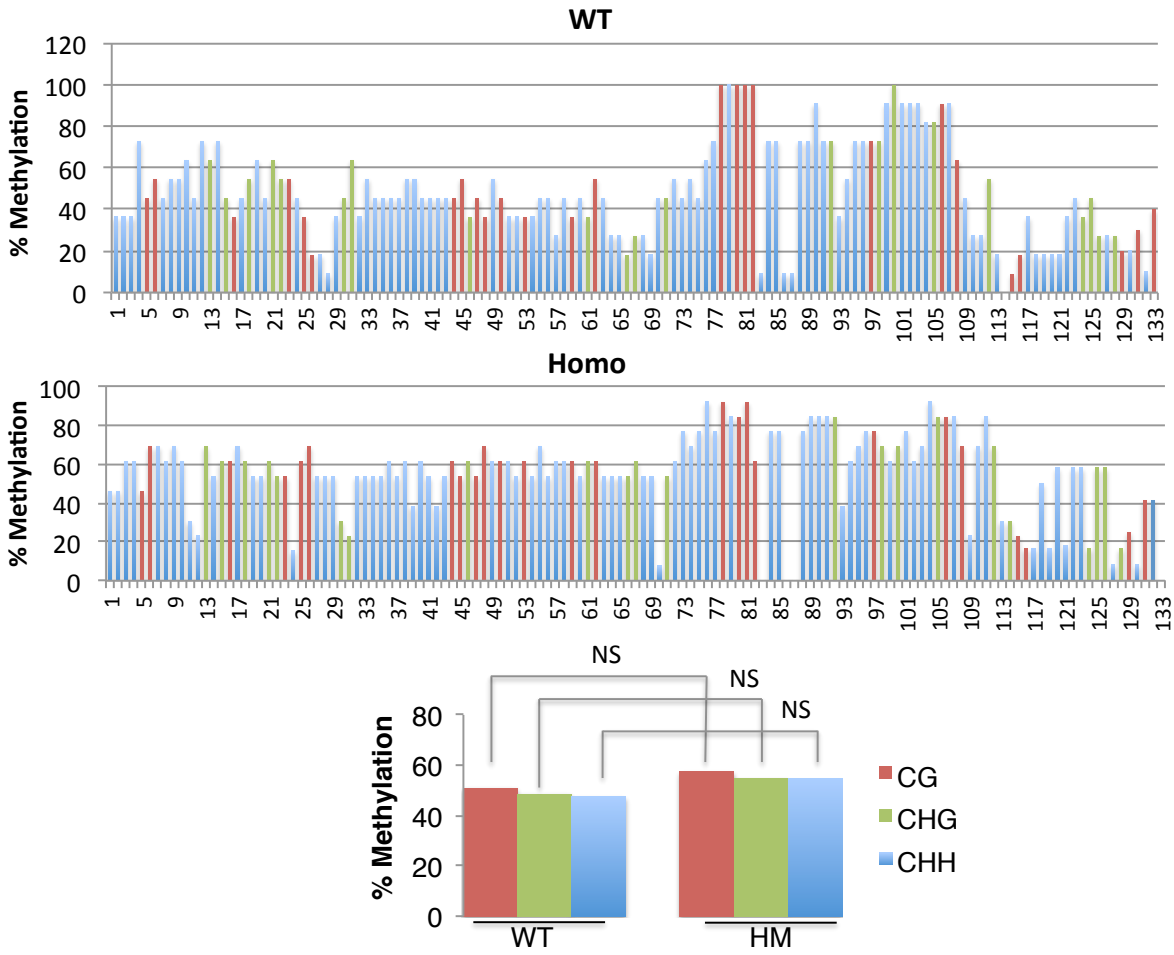


Fig. 12. DNA methylation in the promoter region of homozygous *CA-gOsRac1*. Methylation status of homozygous (A) and wild type (B) plants was assessed by bisulfite sequencing of 1kb-fragment from the transcription start point in the promoter region of *OsRac1*. Three plants each for the homozygous and wild type plants were treated with bilsulfite, amplified by nested PCR and cloned in to Topo blunt vector. Sixteen colonies from each plant were sequenced to evaluate the methylation levels. The percentage of methylation at each cytosine pattern (CG, CHG, and CHH) was calculated by the formula: number of methylated colonies/total number of sequenced colonies x 100%. (C) Mean values of DNA methylation at the three cytosine patterns in homozygous and wild type measured from (A) and (B). NS: no significant difference between indicated samples (Student's t-Test, P>0.05).

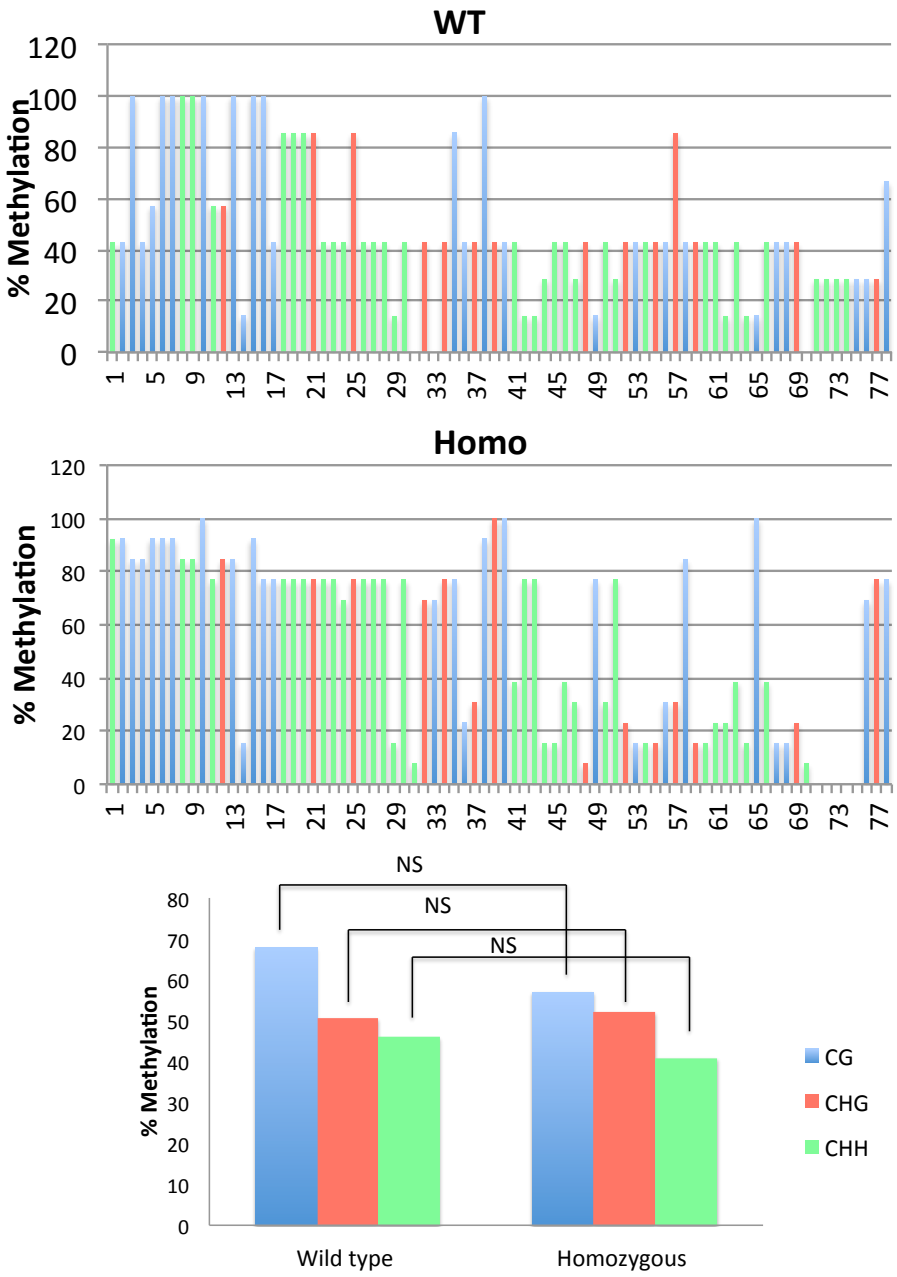


Fig. 13. DNA methylation in the gene body region of homozygous *CA-gOsRac1*. Methylation status of homozygous (A) and wild type (B) plants was assessed by bisulfite sequencing of 400bp-fragment surrounding *loxP*. (C) Mean values of DNA methylation at the three cytosine patterns in homozygous and wild type measured from (A) and (B). . NS: no significant difference between indicated samples (Student's t-Test, P>0.05).

Chapter 4

DISCUSSION

In this study, we generated *CA-gOsRac1* mutant rice plants by homologous recombination-based gene targeting and removal of the hygromycin resistant marker by using the Cre-*loxP* specific recombination. Resultant homozygous *CA-gOsRac1* rice plants exhibited immune responses in the absence of pathogen infection, although expression of the targeted *CA-OsRac1* gene was low in some tissues.

4.1. Generation of *OsRac1* G19V point mutation by HR-mediated gene targeting

OsRac1-G19V was generated by the similar HR-mediated gene targeting procedure applied to create knock out mutants of *Adh2* (Terada et al., 2007), *Waxy* (Terada et al., 2002), *OsDRM2* (Moritoh et al., 2012), knock in mutant of *OsMET1a* (Yamauchi et al., 2009) and various substitution mutations in *Adh2* (Jhozuka-Hisatomi et al., 2008). Compare to these previous studies, the frequency of *OsRac1* gene targeting showed 5-10 times increase in recombination frequency (Table 1). According to Terada et al (2007), the survived calli upon positive-negative selection are mainly attributed to true homologous recombination and border independent random integration. The latter event constitutes the lost of *DT-A*, followed by random integration of targeting vector. Therefore, we speculate that the high efficiency of gene targeting on *OsRac1* was due to the possible reduced border independent random integration event compared to true homologous recombination. Although the homologous recombination frequency of vector B was higher than vector A, the efficiency of mutant transfer was found higher in vector A compared to vector B (Table 1). This result was corresponded to our

observation in the case of generating various mutants in *Adh* gene, in which the nucleotide mutant near to the *hpt* marker showed higher transfer efficiency than the ones far from *hpt* (Johzuka-Hisatomi et al., 2008). This result indicates the importance of homologous sequence designed for gene targeting.

In attempt to remove exogenous marker, two main methods have been applied to introduce Cre-*loxP*: Cre transient expression and chemical-induced Cre expression. The former was performed by sexual cross (Odell et al., 1990), further round of transformation upon positive-negative selection (Gleave et al., 1999) and delivery of Cre fused with nuclear localization signal (NCre) under the control of highly activated promoter into targeted *waxy* mutant cells by particle bombardment without any selection marker (Terada et al., 2010). These methods were shown to cause toxic effect to the cells due to a long time exposition to genomic DNA. Furthermore, the efficiency of N-Cre bombardment method developed by Terada group (2010) was very low and the results are not enough to confirm the recombination was exactly due to NCre. Our study employed β-estradiol inducible Cre method for positive marker elimination. This was found more efficient, possible to eliminate positive selection marker by PCR screening, and reducing the toxic effect of Cre due to continuous exposition to genomic DNA compared to transient expression system used in previous studies.

Overall, HR-mediated gene targeting with positive-negative selection followed by the "clean up" of exogenous marker using in our research opens up the ability to create various mutants of interest. This method, thereby, appears to be a powerful tool in generating materials for studying gene function and crop improvement.

4.2. Low expression of *CA-gOsRac1* at the mRNA level

It could be argue that the location of *loxP* in the first intron of *CA-gOsRac1* may disrupt the splicing pattern of the mRNA *CA-gOsRac1* and/or affect the stability of the mRNA. However, the northern blotting assay indicated that no extra spliced forms of the mRNA was detected in homozygous *CA-gOsRac1* (Fig. 10), excluding the possible interference of *loxP* on splicing pattern of the mRNA *CA-gOsRac1*. The stability of mRNA *OsRac1* will be addressed in the future study by nuclear run-on assay.

Real-time PCR analysis showed that mutant transcript containing single *loxP* was not suppressed as observed in the case of *loxP* plus mutation (Fig. 9), plausibly suggesting that constitutively active mutation G19V may have effect on *OsRac1* expression. Our results showing that of the 5 GT lines obtained only one produced viable mature plants and seeds may support this hypothesis (Table 1). This observation corresponds to the phenotype of *CA-OsRac1* generated from our early study, in which some *CA-OsRac1* lines did not produce seeds, probably due to the effect of constitutively active OsRac1-G19V mutation. Another relevant observation is that after removal of the *hpt*-ΔEn fragment from the targeted *OsRac1* locus through Cre-*loxP* recombination it became difficult to obtain shoots from the callus. Since *CA-gOsRac1* expression is initiated after removal of the *hpt-ΔEn* fragment from the targeted *OsRac1* locus. This observation suggests that *CA-gOsRac1* may have had a harmful effect on shoot growth. In addition, although the mutant transcript was expressed at low level, it still exhibited slightly dwarf phenotype for the homozygous *CA-gOsRac1* (Fig. 5C), probably due to the effect of constitutively active mutation G19V. This hypothesis was further supported by a slightly reduced expression of *OsRac1* mRNA in wild type plants upon chitin treatment (data not shown), suggesting that *CA-OsRac1* was regulated at the mRNA level. If wild type level of *CA-OsRac1* expression under the control of the native promoter is harmful

for the growth of plants it is possible that only targeted plants, which showed very low level of CA-gOsRac1 expression, were recovered. This possibility will be tested in future studies.

The methylation analysis of both *OsRac1* promoter and gene body regions demonstrated that methylation status was not likely the reason for suppressed *OsRac1* expression. It is necessary to check the methylation status in the whole gene body region. However, although the tested region showed the high density of methylation (400 bp contains 77 methylated positions), there was no significant difference in methylation status was detected. Probably, methylation status did not affect gene expression. Therefore, at present, the reasons for low expression of homozygous *CA-gOsRac1* at the mRNA remain elusive. It may due to the detrimental phenotype effect of constitutively active *OsRac1*, the location of *loxP*, or other unknown factors. In recent reports on rice plants with nucleotide substitution by gene targeting, no suppression of mutant transcript was detected (Endo et al., 2007, Wakasa et al., 2012, Saika et al., 2011). Therefore, although the mechanisms of suppression of *CA-OsRac1* expression in the transgenic rice plants remain unclear, this is not a universal case of base substitution mutants generated by homologous recombination.

4.3. Homozygous *CA-gOsRac1* mutation induces downstream signaling in rice immunity

Although homozygous *CA-gOsRac1* showed the low expression at the mRNA level, some characteristics of *CA-gOsRac1* in innate immunity was identified including the activation of pathogen related genes (Fig. 7A), induction of hypersensitive response cell death (Fig. 7B) and stimulation of various downstream genes known to be induced by *M. oryzae* and/or *Xoo* infection (Table 3). The induction of *PAL1* and *PBZ1* by *CA-OsRac1* has been previously shown (Thao et al., 2007, Chen et al., 2010, Akamatsu et al., 2013). Hypersensitive response-

like cell death was previously detected in the leaf sheath of *CA-OsRac1* plants after infection with a compatible blast fungus (Ono et al., 2001). In addition, results of the microarray analysis proved the importance of *OsRac1* in rice immunity by inducing various transcripts that play critical role in immune responses such as members of WRKY transcription factors, MAP kinase pathway, pathogen-related genes. Unfortunately, we failed in isolating *CA-OsRac1* protein by pull-down assay probably due to the low expression of *CA-gOsRac1* protein rendered by suppressed *OsRac1* mRNA. Therefore, the induction of downstream genes by *CA-gOsRac1* was limited at the mRNA level. Leaf sheath infection with a compatible rice blast fungus was shown to induce hypersensitive response-like cell death. However, we were not able to find enhanced disease resistance phenotype in the homozygous *CA-gOsRac1* plants (Fig. 9). We speculate that the expression level of *CA-gOsRac1* was too low to confer enhanced disease resistance to the transgenic plants.

In order to overcome the low expression of *OsRac1* in homozygous *CA-gOsRac1*, two approaches will be addressed in future studies to establish rice plants that enhance disease resistance. If the location of *loxP* interferes with *OsRac1* expression, the completed removal of selection marker without leaving *loxP* footprint in *OsRac1* locus is required. Recently, transposable elements such as Ac/Ds have been recruited as a strategy to eliminate completely marker genes without leaving any footprint in tomato (Goldsbrough et al., 1993) and rice (Cotsaftis et al., 2002). In principle, the marker is placed within the mobile sequence; and its removal depends on the excision of Ac element. In the second approach, we aimed to generate GEF1 mutation, an upstream signal of OsRac1. A substitution of Serine residues S549 by phosphomimic aspartic acid (D) in GEF1 is found to change OsRac1 from the GDP- to GTP-bound form (Akamatsu et al., 2013). In fact, we successfully generated *OsGEF1* mutation by

the same gene targeting procedure applied for *OsRac1* gene. Interestingly, *GEF1-S549D* mutation was not suppressed at the mRNA level (Kawano et al., un-published data). At present, disease resistance of *OsGEF1*-S549D mutation plants is being examined.

In summary, we successfully generated endogenous CA-mutation under the control of the native promoter by using homologous recombination-mediated gene targeting and Cre-*loxP*-specific recombination. Although, the low expression level of *CA-gOsRac1* limited further study of the transgenic plants for disease resistance, the strategy of gene targeting shown in this report is useful for generating various defined mutations in the rice genome.

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Table S1 List of primers for PCR cloning, PCR analysis and DNA sequencing analyses

Designation	Sequences (5' --->3')[a]	Designation	Sequences (5' --->3')	Comments
PCR cloning of homology arms in targeting vectors				
Rac1 5' 1st-F	GGCCAGTTCATCGACGGATT	Rac1 5' 1st-R2	CAACTGGCCTTGTTCTTTTCGAG	The first round of nested PCRs for cloning of 5'-homologous regions of pOsRac1G19V
Rac1 5'-F	AGTCTTAATTAAAATTTGCACATCTCAAGTCCATTTTCCTTTC	Rac1 5'-R	TGACGCCCGGGCATTAGCGGCGGCGCGGCACTCAACGGTGGG	The second round of nested PCRs for cloning of 5'-homologous region of pOsRac1G19V
Rac1 3' 1st-F	ATGATGGCAAGTTCAAACGG	Rac1 3' 1st-R2	AGTGATGGTTGCTCCATCACATTTGTTCGG	The first round of nested PCRs for cloning of 3'-homologous regions of pOsRac1G19V
Rac1 3'-F	AGTCGTTTAAACCCCTTCCTCTCCGTTCTTGCTCCGGCCCGC	Rac1 3'-R	TGACGGCGCGCCCTTGTTTGTACAAGGAGAAATGTGTAACCT	The second round of nested PCRs for cloning of 3'-homologous region of pOsRac1G19V
Introduction of G56T nucleotide sustitution				
Rac1 G19V-F	CACCGTGGGGGACGTCGCGGTGGGGAAGAC	Rac1 G19V-R	GTCTTCCCCACCGCGACGTCCCCCACGGTG	Introduction of G56T nucleotide substitution into 5'-homologous regions of pOsRac1G19V
PCR screening of GT transformants				
A5-F	ACCAAAACACTAGTCCACCTACAAG	A5-R	CAAAAGCGAATAAACTTAAAAACCA	Screening of GT transformants generated with pOsRac1G19V (Figure 1B, S1A)
A3-F	CTCACCAGGATCAGTACGTTAAGTT	A3-R	GGAGGGCTTTGCTGGATCTGCTGTAAGGTA	Screening of GT transformants generated with pOsRac1G19V (Figure 1B, S1A)
PCR Screening of marker-free transformants				
ACL-F	ACCATCATTAACACGCCATACTT	ACL-R	GTGAGCTTGAGCTAGCTTTCCC	Detection of the elimination of *hpt-ΔEn* fragment from *OsRac1* locus transformed with pOsRac1G19V-A (Figure 1C, S1B)
H1F	GTATAATGTATGCTATACGAAGTTATGTTT			Detection of *loxP* site
Primers for sequencing analysis				
Rac1 5' Seq F-1	GATTTAATGGAAGCCACCTT			
Rac1 5' Seq F-2	TGTGCGGATATACACGGCTA			
Rac1 5' Seq F-3	GTAGTTTACTGATGGATTGT			
Rac1 5' Seq R-1	GGCCTAAAGTTTTAACGTGT			
Rac1 5' Seq R-2	GCTTCCTGCACATGGTGATG			
Rac1 5' Seq R-3	TTTGAGCATGGAGCAAGTAA			
Rac1 5' Seq R-4	CGTCAAATTTAGGCTATTGGATCTCATACA			
Rac1 5' Seq R-5	GAATATATAAAAAGGGATAA			
Rac1 3' Seq F-1	GAGCCGATGTGTTCATCCTG			
Rac1 3' Seq F-2	GTTGGAACCAAGTTGGGTAT			
Rac1 3' Seq F-3	GTAAGGTACCTTTGAAGTTT			
Rac1 3' Seq F-4	GGAAACTACTGGACCTAACAGACCTAACAG			
Rac1 3' Seq F-5	TAACACATCAATCTAGAAGT			
Rac1 3' Seq F-6	CTGATTGTAGTAAAATCAGC			
Rac1 3' Seq R-1	GTTTCTTGTAATCCATTCAT			
Rac1 3' Seq R-2	TGAACATCATGGTGATGGAA			
Rac1 3' Seq R-3	TCCCTAACATGTAGAAAGAG			
Rac1 3' Seq R-4	GGCTTTCTTGTCTCTAGGTA			
Primers used for real time PCR				
OsRac1-F	ACAACGGAGCAGGGAGAAGAAC	OsRac1-R	ACATCCTTATGTCTTGGAGGTTG	
PAL1-F	TGAATAACAGTGGAGTGTGGAG	PAL1-R	AACCTGCCACTCGTACCAAG	
PBZ1-F	ATGAAGCTTAACCCTGCCGC	PBZ1-R	GTCTCCGTCGAGTGTGACTTG	
Ubiquitin-F	AACCAGCTGAGGCCCAAGA	Ubiquitin-R	ACGATTGATTTAACCAGTCCATGA	
AK107199-F	CGACGGAAATTGTAGTTTAGGG	AK107199-R	CTTTCCTCCCATCTCCAGTG	WRKY transcription factor 24
AK106282-F	GGCGCCGATTAAGGTTCTC	AK106282-R	CATCTCGGTCAGCTTCTTGTTCT	Similar to WRKY transcription factor 28
AK111606-F	AAGGCTTGAGCTTCTTCTTCC	AK111606-R	TGCTTGACAGAAACCAGCAA	WRKY transcription factor 69
AK112056-F	CCTCTTGGGTCGATCTCTTG	AK112056-R	CCTTCTTTCGTTTCCCATCA	Similar to Y19 protein
AK073812-F	CAATGCACTGCTCCAAGAAA	AK073812-R	CCTCACCACATTTCACAAACA	Similar to ethylene-responsive transcription factor 5 (ERF5)
AK073848-F	ATCTGGCAAGCATGATTCTG	AK073848-R	GAACAAAAGGAGATGTTTTGCAG	NAC domain-containing protein 68 (OsNAC4)
AK068725-F	ACGAGGAAAGAGGCTGTTGA	AK068725-R	TCTGCTAGGGAAGCGCTAAA	mitogen-activated kinase kinase kinase alpha (MAPKKK3)
AK100082-F	GGCAGCTTGGACAGCATAGT	AK100082-R	TACATGCAAATAACTGAAGGATGG	Receptor-like protein kinase 5 precursor
AK107566-F	TATGCAGCCCAATGCTTGTA	AK107566-R	TGCTTTAGCCAAAATATCTGGA	Leucine rich repeat, N-terminal domain contianing protein
AK066825-F	CTGTACTTCGCCTCCTCGTC	AK066825-R	ACAACAACTCGCTGCCTACC	Similar to lipoxygenase, chloroplast precursor
AK067667-F	ATGGGTGCAGTGAGCAGAA	AK067667-R	GGAAGCAGTCGTGGAAGAAG	Peroxidase
AK100973-F	AGCCCCCAAGCCTGAGTAAT	AK100973-R	GTACACCATGTGGGTTGTGC	Acidic class III chitinase Oschib3a precursor
MgPot2-F	ACGACCCGTCTTTACTTATTTGG	MgPot2-R	AAGTAGCGTTGGTTTTGTTGGAT	
Primers used for bisulphite sequencing				
OsRac1pro1-F	ATGAGTAAAAGTGTAACGGGGAGT	OsRac1pro1-R	CACTCTAGCTCTGCCTCCAGCTCC	The first round of nested PCR in the promoter region of OsRac1
OsRac1pro2-F	GTGCTAAAATAGTTTTCATATATATT	OsRac1pro2-R	AACTACAAAATTAATAAATACAATCAAC	The second round of nested PCR in the promoter region of OsRac1
OsRac1-genebody-F	TGTGAGAGTGAACAAGAGATAAGC	OsRac1-genebody-R	AGCTTGAGCTAGCTTTCCCCAC	
Primer used for Northern Blotting				
OsRac1-F	AGATAGGGCCTATCTTGCTGATCATC	OsRac1-R	CTAGAAGTTTCCTCCTAGCTGCAAGC	
Actin1-F	CTTCGGACCCAAGAATGCTA	Actin1-R	CTTCGATGTTATTCATTTCATAG	

a: The underlined nucleptides represent cleavage sites for restriction enzymes, *Pac*I or *Srf*I, respectively