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The phenome analysis of mutant alleles in *Leucine-Rich Repeat Receptor-Like Kinase* genes in rice reveals new potential targets for stress tolerant cereals

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ABSTRACT

Plants are constantly exposed to a variety of biotic and abiotic stresses that reduce their fitness and performance. At the molecular level, the perception of extracellular stimuli and the subsequent activation of defense responses require a complex interplay of signaling cascades, in which protein phosphorylation plays a central role. Several studies have shown that some members of the Leucine-Rich Repeat Receptor-Like Kinase (LRR-RLK) family are involved in stress and developmental pathways. We report here a systematic analysis of the role of the members of this gene family by mutant phenotyping in the monocotyledon model plant rice, *Oryza sativa*. We have then targeted 176 of the ~320 *LRR-RLK* genes (55.7%) and genotyped 288 mutant lines. Position of the insertion was confirmed in 128 lines corresponding to 100 *LRR-RLK* genes (31.6% of the entire family). All mutant lines harboring homozygous insertions have been screened for phenotypes under normal conditions and under various abiotic stresses. Mutant plants have been observed at several stages of growth, from seedlings in Petri dishes to flowering and grain filling under greenhouse conditions. Our results show that 37 of the *LRR-RLK* rice genes are potential targets for improvement especially in the generation of abiotic stress tolerant cereals.

1. Introduction

Nowadays, rice of Asian origin (*Oryza sativa* L.) is the staple food for more than half of the human population. In less than 40 years, the world's population is predicted to reach 9 billion, raising the so-called "9-billion-people" issue [1]. For sustainable rice production in the years to come, a number of challenges need to be addressed by the entire rice community with the common goal of creating new elite rice varieties [2,3]. Large efforts have focused in the last years to complete sequencing of several *Oryza* genomes [4–10]. In functional genomics, the challenge is now to system-atically assign a biological function to all genes in the genomes. To help in this task, the rice community worldwide has started to share efforts in the late 90's to produce insertion mutant collections required for gene functional analyses [11,12]. These mutant collections are available in several laboratories around the world: CSIRO in Australia [13], NIAS in Japan [14], OSTID in Europe [15], OTL in France [16], POSTECH [17] and PMBBRC [18] in Korea, RMD in China [19], TRIM in Taiwan [20], and UCD in USA [21]. These mutant collections contain insertion lines created with T-DNA, *Tos17*, *Ds*, and *dSpm* inserts mutagens and the engineered mutagens may additionally carry gene traps, enhancer traps and/or activation tags. They have been generated in different cultivars: Nipponbare (NB), DongJin (DJ), HwaYoung (HW), Zhonghua 11 (Z11), Zhonghua 15, Tainung 67 (TNG) and Kitaake. All these lines are listed based on their flanking sequence tags (FSTs) in two databases: RiceGE (http://signal.salk.edu/cgi-bin/RiceGE) and Ory-GenesDB (http://orygenesdb.cirad.fr). In total, ~225,000 FSTs are precisely positioned on the ssp. *japonica* cv. Nipponbare sequence

Table 1
List of LRR-RLK genes with known functions in rice.

Accession numbers	Names	Orthologous relationships described	Functions/comments	References
Os11g36180	Xa21		Resistance to *Xanthomonas oryzae* pv. *oryzae*	[61]
Os02g34790	Xoo-INDUCED KINASE 1 (XIK1)		Positively regulates XA21-mediated immunity	[62]
Os11g47000	Xa3/Xa26		Immune receptor playing the same role as Xa21	[63,64]
Os04g52780	*Os* FLAGELLIN SENSING 2 (OsFLS2)	AtFLS2	As in *Arabidopsis* mediates flagellin perception	[65]
Os01g52050	*Os* BRASSINOSTEROID INSENSITIVE 1 (OsBRI1)	AtBRI1	Cell elongation and cell division in shoot	[66]
Os09g12240	Os BRI1-LIKE 1 (OsBRL1)	AtBRL1	Cell elongation and cell division in shoot and root in conjunction with OsBRI1	[67]
Os08g25380	Os BRI1-LIKE 3 (OsBRL3)	AtBRL3	Cell elongation and cell division in shoot and root in conjunction with OsBRI1	[67]
Os06g50340	FLORAL ORGAN NUMBER 1 (FON1)	AtCLAVATA1 (AtCLV1)	Regulates floral meristem size	[68]
Os01g68870	MULTIPLE SPOROCYTE 1 (MSP1)	At EXTRA SPOROGENOUS CELLS / EXCESS MICROSPOROCYTES1 (EXS/EMS1)	Necessary to restrict the number of cells entering into male and female sporogenesis and to initiate anther wall formation	[55]
Os02g10100	MSP-LIKE 1 (MSL1)	At EXTRA SPOROGENOUS CELLS / EXCESS MICROSPOROCYTES1 (EXS/EMS1)	Necessary to restrict the number of cells entering into male and female sporogenesis and to initiate anther wall formation in conjunction with MSP1	[69]
Os03g12730	BLAST RESISTANCE-RELATED (BRR1)	At BARELY ANY MERISTEM (BAM1 and BAM2)	Involved in blast resistance	[70]
Os03g50810	*Os* TRANSMEMBRANE KINASE (OsTMK)	four members of the TRANSMEMBRANE KINASE (TMK) subfamily	Role in plant growth	[71]
Os04g38480	*Os* SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE 2 (OsSERK2)	AtSERKs	Required for both Xa21, Xa3/Xa26 and FLS2 signaling and bassinosteroid-regulated plant growth	[72]
Os08g07760	*Os* SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE 1 (OsSERK1) also named Os BRI1-ASSOCIATED KINASE 1 (OsBAK1)	AtSERKs	Functions in rice development, affecting growth and angle of lamina joint; brassinosteroid signaling?	[73–75]
high degree of similarity but not identical to Os08g07760	BENZOTHIADIAZOLE-INDUCED SOMATIC EMBRYOGENESIS RECEPTOR KINASE 1 (BISERK1)	AtSERKs	Up-regulated upon *Magnaporthe* inoculation	[76]
Os08g34380	COMMISSURAL VEIN EXCESSIVE 1 (COE1)	AtSERKs	Responsible for commissural vein pattern formation in rice	[77]
Os02g14120	DEFECTIVE IN OUTER CELL LAYER SPECIFICATION 1 (DOCS1) also named OsSERK-like 4 (OsSERL4)		Involved in the proper development of root outer cell layers	[75,78,79]
Os02g40240	LEAF PANICLE 2 (LP2)		Negative regulator in drought response	[80,81]
Os02g12440	GAMMA-RAY INDUCED LRR-RLK 1 (GIRL1)		Highly induced by gamma irradiation, by several abiotic stresses (salt, osmotic, and heat), by hormonal treatment with salicylic acid or abscisic acid, but downregulated in response to jasmonic acid treatment	[82]
Os05g40770	OsRPK1		A salt-responding protein, whose expression is also induced by cold, drought, and abscisic acid; affects root architecture by negatively regulating polar transport and accumulation of auxin in roots	[83,84]
Os07g41140	RECEPTOR-LIKE PROTEIN KINASE 1 (RPK1)	AtRPK1	Overexpression of both Arabidopsis and rice RPK1 receptors induces a reduction in salt tolerence in Arabidopsis transgenic plants	[85,86]
Os06g03970	STRESS-INDUCED PROTEIN KINASE GENE 1 (OsSIK1)		Affects stomatal density in leaf epidermis and plays important roles in salt and drought stresses	[87]
Os04g48760	XIAO ("small" in Chinese)		Regulates brassinosteroid signaling and cell division	[88]
Os11g07225-like1 and Os11g07225-like2	25L1 and 25L2		Specific to wild *Oryza rufipogon* rice; responsible for the high temperature-dependent expression of hybrid weakness	[89]
Os02g05980	LEUCINE-RICH REPEAT RECEPTOR-LIKE KINASE 1 (LRK1)		Cluster of 8 genes; LRK1 present in Dongxiang wild rice, but absent in Guichao2; Overexpression of LRK1 improved quantitative yield components	[90]

Search in international
mutant collections

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Lines selection

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Genotyping

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Homozygous plants

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Phenotyping on MS/2 medium → Mutant plants with phenotype = putative LRR-RLK involved in leaf or root growth

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Mutant plant w/o phenotype

|

Phenotyping on mannitol and salt medium

↓

Mutant plants with phenotype = putative LRR-RLK involved in conditional response to abiotic stress

Fig. 1. Summarized schematic representation of our screen strategy.

(MSU v7.0 in OryGenesDB) with ∼125,000 located in the ∼35,000 genic regions (i.e., an average of 3.6 FSTs/locus) [12,22–25].

Leucine-Rich Repeat Receptor-Like Kinases (*LRR-RLKs*) belong to the largest subfamily among the *Receptor-Like Kinase* (*RLK*) genes [26–28]. These receptors are important mediators of cell-to-cell communication to relay developmental cues and environmental stimuli or to activate defense/resistance against pathogens in plants [29–33] (for reviews see also the special issue of JIPB dedicated to Receptor-Like Kinases in Dec. 2013). In *Arabidopsis*, to date, a function has been assigned to ∼35% of the ∼230 LRR-RLK members. The most studied receptors are *BRASSINOSTEROID INSENSITIVE 1* (*BRI1*), a receptor for the brassinosteroid hormone [34]; *ERECTA*, a pleiotropic regulator of many developmental processes and responses to biotic and abiotic stimuli [35–37]; *CLAVATA1* (*CLV1*) controlling shoot and floral meristem homeostasis [38]; *FLAGELLIN SENSING 2* (*FLS2*), a gene participating in the perception of the bacterial elicitor flagellin and *EF-TU RECEPTOR* (*EFR*), the receptor of the bacterial elongation factor Tu (EF-Tu), which both confer broad-spectrum bacterial resistance in *Arabidopsis* [39,40]; and receptors belonging to the *SERK* subfamily (*SERK1*, *SERK2*, and *SERK3*), which are described as co-receptors in multiple signaling pathways, notably *BRI1*, *FLS2*, and *EFR* pathways [41–46]. The rice genome has been shown to contain ∼320 *LRR-RLK* genes and a function has been assigned to less than 10% of them (Table 1) [47,48]. Because of their many roles in developmental and stress responses, *LRR-RLK* genes are promising targets for crop improvement [49].

In an attempt to identify new rice *LRR-RLK* genes involved in stress tolerance, we carried out a reverse genetic approach [50]. We generated a collection of homozygous insertion mutant lines for ∼35% of the whole *LRR-RLK* gene family without preconceived ideas about putative gene functions. These mutant plants have been screened *in vitro* for altered growth phenotypes at the seedling stage under control and abiotic (salt and mannitol) stress conditions. We looked particularly for mutants with conditional developmental phenotypes under abiotic stress. Our strategy is summarized in Fig. 1. Our analysis reveals new uncharacterized *LRR-RLK* genes putatively involved in abiotic stress responses. These genes are potential targets for breeding of salt- and drought-tolerant cereals.

2. Material and methods

2.1. Plant material and genotyping

Accession numbers of the mutant lines to be genotyped were defined on OryGenesDB (http://orygenesdb.cirad.fr/). Seeds were ordered to OTL, NIAS, Postech, RMD, and TRIM. Upon receipt, when available, 15–20 T1 or T2 seeds were sown in the greenhouse (28 °C, 60% humidity, 16:8 photoperiod). Some of these mutant lines have been genotyped by Southern blotting as described previously [51], others by a quick direct PCR method following manufacturer instructions (Phire® Plant Direct PCR Kit, Finnzymes). For Southern blots, genomic DNA was extracted from leaves of 4 week-old plants. Briefly, tissues were freeze-dried overnight and disrupted the next day with a mixer mill. Powder was mixed with extraction buffer (Tris–HCl 200 mM (pH 7.5), EDTA 25 mM (pH 8.0), 0.025% SDS, and NaCl 25 mM) and precipitated with isopropanol. Eight micrograms of genomic DNA were digested with restriction enzymes and loaded on a 0.8% agarose gel for electrophoresis at 25 V for ∼17–18 h. DNA was transferred on nylon membrane and hybridized with radioactive probes labelled by the random-prime method. For Southern probes and PCR-based genotyping, primers were designed on the OryGenesDB web site (http://orygenesdb.cirad.fr/tools.html). For Southern blots, 2 probes were generated by PCR: a gene-specific probe (chosen, depending on the restriction enzyme used, to hybridize to a DNA fragment <12 kb) and a vector-specific probe (HPT or Tos17). For lines genotyped by PCR, we used 2 pairs of primers. The first pair, gene-specific, to amplify a DNA fragment surrounding the insertion; and the second one, using one gene-specific primer and one T-DNA- or Tos17-specific border primer.

2.2. Growth conditions for mutant screen

In all experiments, 10 seeds of T2 or T3 plants were grown vertically in sterile square Petri dishes (Corning, 431.301; 20 cm × 20 cm) under controlled conditions (day/night temperature of 28/25 °C, a 12 h photoperiod, and a light intensity of 500 μEm^{-2} s^{-1}) as described previously [52]. Briefly, after sterilization, the seeds were sown on square Petri dishes containing 250 mL of half strength Murashige and Skoog (MS/2) solid medium with the radicle oriented downwards. The MS/2 solid medium was composed of 2.15 g L^{-1} of Murashige and Skoog medium basal salt mixture (Duchefa Biochemie, M0221), 75 mg L^{-1} Murashige and Skoog vitamin mixture (Duchefa Biochemie, M0409) and 8 g L^{-1} of agarose type II (Sigma–Aldrich, A6877). For salt and mannitol medium, 7 g L^{-1} of NaCl (120 mM) and 21.9 g L^{-1} of mannitol (120 mM), respectively, were added to MS/2 medium before autoclaving. After 6 days of growth, the lengths of the seminal root and second leaf (i.e., the leaf following the first incomplete leaf) were recorded for each of the 10 plantlets.

3. Results and discussion

3.1. More than 90% of the LRR-RLK genes are putatively tagged by one insertion in international collections

In this study, we used the method we described previously to establish our *LRR-RLK* gene set [53]. Briefly, the hmmsearch program was run to extract peptide sequences containing both LRRs and a kinase domain (data not shown) [54]. We ran the program on the MSU version 7.0 of the Nipponbare genome and compared our gene list with the one published previously [25,47,48]. We kept a list of 316 *LRR-RLK* genes considered for mutant analysis (Suppl. Table 1). These genes are unequally distributed on the 12 chro-

Table 2
Number of LRR-RLK genes and clusters per chromosome.

Chromosome	Sequence length (bp)	Number of non-TE[a] genes	Number of LRR-RLK genes	Number of LRR-RLK genes per Mb	Number of LRR-RLK genes for 1000 non-TE loci	Proportion of LRR-RLK genes per chromosome	Nb of clusters	Nb of genes in clusters	Mean number of genes per cluster
1	43,270,923	5078	34	0.79	6.7	10.8	5	11	2.2
2	35,937,250	4143	43	1.20	10.4	13.6	7	26	3.7
3	36,413,819	4388	19	0.52	4.3	6.0	1	2	2.0
4	35,502,694	3419	23	0.65	6.7	7.3	2	8	4.0
5	29,958,434	3118	26	0.87	8.3	8.2	3	10	3.3
6	31,248,787	3236	36	1.15	11.1	11.4	5	20	4.0
7	29,697,621	3065	17	0.57	5.5	5.4	1	2	2.0
8	28,443,022	2762	25	0.88	9.1	7.9	3	10	3.3
9	23,012,720	2260	20	0.87	8.8	6.3	3	8	2.7
10	23,207,287	2298	15	0.65	6.5	4.7	3	6	2.0
11	29,021,106	2707	46	1.59	17.0	14.6	6	35	5.8
12	27,531,856	2443	12	0.44	4.9	3.8	1	2	2.0
Total	373,245,519	39,102	316	0.85	8.1	100	40	140	3.5

[a] TE: Transposable elements.

mosomes, with chromosomes 2, 6 and 11 comprising ∼40% of the 316 genes (Table 2). Moreover, many of these genes (140, 44.3%) belong to 40 tandem duplication clusters. These clusters contain 2 to 13 genes (Table 2 and Suppl. Table 1).

We used the OryGenesDB database to identify insertion mutants available in international collections [23,24]. This search revealed that (i) 26 out of the 316 genes (8.5%) had no insertion, (ii) among the 290 genes with at least one predicted insertion, the number of insertions per genes was on average 8.47 +/− 0.75 extending from 1 to 156 insertions (Fig. 2 (Box 1) and Suppl. Table 2). This number is twice as high as the current average number of inserts available per gene in the rice genome (3.6 FSTs/locus), suggesting that some *LRR-RLK* genes are insertion hot spots. To select the mutant lines to be genotyped, we gave first priority to mutants present in our own collection (OTL). We also chose insertions in the coding region or in the promoter within 200 bp of transcription initiation when available. We ended up with 288 mutant lines predicted to tag 176 (55.7%) of the 316 *LRR-RLK* genes. These lines have been identified in the OTL, Postech, RMD, OSTID, UCD, TRIM, and NIAS collections (Suppl. Table 1).

### 3.2. Generation of a collection of 128 insertion lines for LRR-RLK genes

Mutant plants segregating for the mutations were identified by Southern blotting or PCR in the 288 mutant lines (Suppl. Fig. 1). Following this large scale characterization, we concluded that 128 (44.4%) lines (in 100 (31.6%) *LRR-RLK* genes) displayed the predicted insertion (Suppl. Table 3). For the excluded 160 lines, we have been unable to confirm the presence of the predicted insertion in the LRR-RLK gene tagged. Among the rearranged lines, we identified both homozygous and heterozygous mutated plants in 94 lines, but only heterozygous plants in 34 lines (Fig. 2 (Box 2)). In 33 out of these 34 heterozygous lines, the low number of plants genotyped could explain this result. However, in one line, AQYD06 (Os11g47030.1), among the 18 plants genotyped, all adult plants were heterozygous (13 plants) or wild type (5 plants) for the insertion (probability = 3.8e^{-6}). This observation suggests that this insertion may affect an essential developmental process. We also observed that in lines 3A-51728 (Os03g05140.1), 1C-10702 (Os06g45020.1), 2D-00806, and 3D-02697 (both with insertions in Os04g15660.1), and ANZE10 (Os01g68870.1), all homozygous plants were sterile. These observations suggest that these mutations could be involved in reproductive organ development. The latter gene, *MSP1* (Os01g68870.1), has already been described in the literature for its function in floral development, particularly

in male and female sporogenesis and in initiation of anther wall formation (Table 1) [55].

3.3. Six mutant lines are affected in leaf and/or root growth on control medium

For phenotyping, we focused particularly on the 89 lines for which we identified homozygous progeny plants. Altogether, these lines tag a total of 79 genes, including 70, 8, and 1 gene tagged by 1, 2, and 3 independent insertions, respectively (Suppl. Table 4). First, we sowed 10 homozygous seeds per line on a control MS/2 medium in Petri dishes. For each plant, we scored the leaf 2 and seminal root lengths 6 days after germination (Suppl. Fig. 2). In parallel and for comparison, we also analyzed the 5 wild type varieties (NB, DJ, HW, Z11, and TNG) as controls. We observed that 24 homozygous mutant lines (27%) showed a statistically significant difference in leaf 2 and/or root length compared to their respective varietal controls (Dunnett test, $p < 0.05$) (Fig. 2 (Box 3)).

To ascertain that the phenotypes observed were not due to other mutations segregating in the line, we further grew on MS/2 control medium the progeny of either a wild type or, when no wild type was available, of an heterozygous sibling of these mutants (Figure 2 (Box 4)). This second evaluation of the phenotype was done for all 24 lines except one (RGT6318 in Os04g57630.1) for which we only found homozygous plants. By comparing the results obtained in these two experiments for the 23 other lines, we observed that the phenotype observed in 13 out of the 23 (56.5%) of the homozygous lines was also identified in their siblings, suggesting that this phenotype was due to independent mutations segregating in the T2 progeny and not to the mutated *LRR-RLK* gene studied. Rice insertion mutant collections have been produced through transformation of callus cultures. The presence of mutations induced by this *in vitro* phase has been well documented [56–59]. Thus, from this screen on control MS medium, we concluded that among the 89 homozygous lines analyzed, 14 (15.7%) harbored a phenotype not linked to the gene under study (Suppl. Table 4). For the 10 other lines, we compared leaf 2 and root lengths of the homozygous plants to those of their null-segregant siblings (Fig. 2 (Box 5)). Our results showed that only 6 lines (*i.e.*, 6.7% of the 89 lines) actually exhibited a phenotype linked to the LRR-RLK mutation (Student test, $p < 0.05$). These 6 lines presented statistically different phenotypes from both their varietal control and their null-segregant siblings. For the 4 other lines, even if their phenotype was slightly statistically different from the varietal control, this difference was not statistically different from their azygous siblings. The 6 *LRR-RLK* genes tagged in these lines presented phenotypes
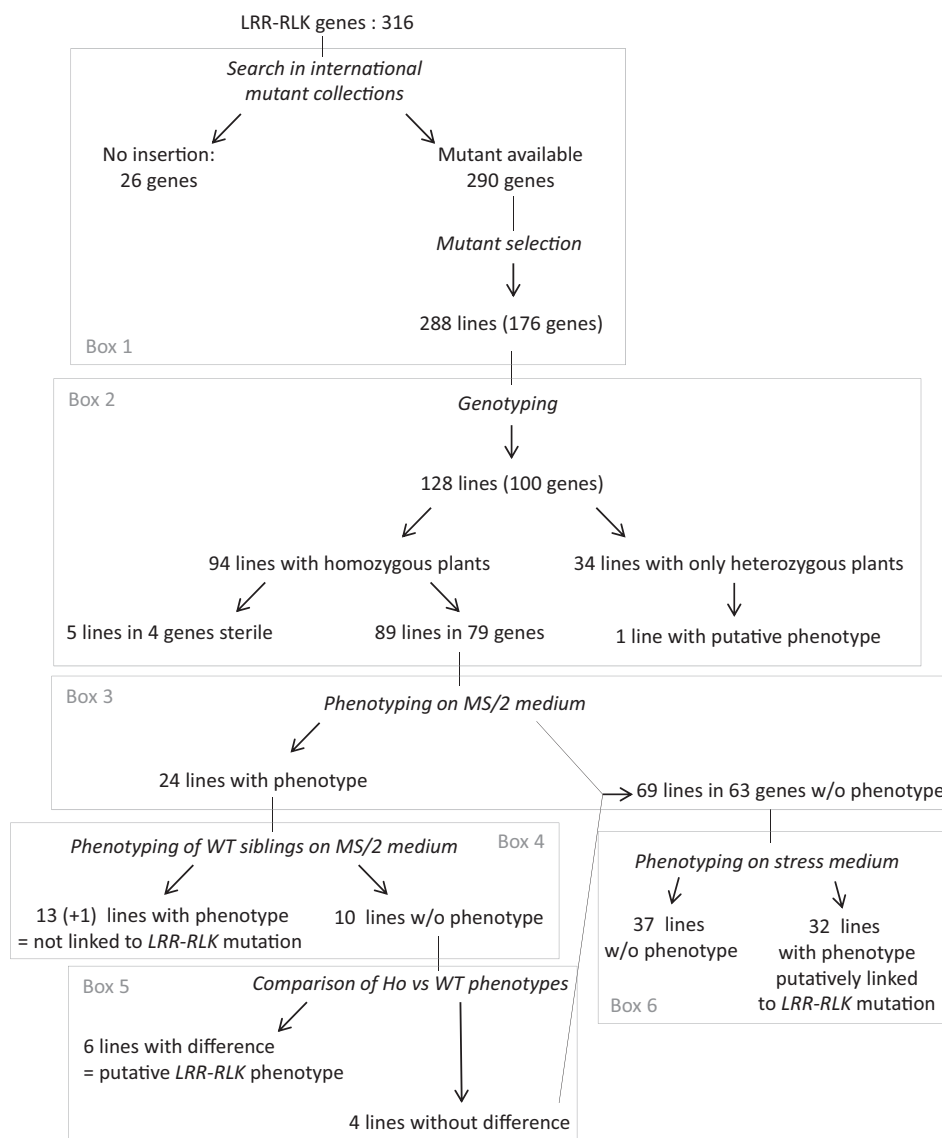
Fig. 2. Detailed view of each step of the mutant screen.

in leaf 2 or root growth (Fig. 3). Among the 5 lines affected in leaf 2 growth, 3 were longer (Os01g60060.1, Os01g60670.1, and Os02g13410.1) and 2 shorter (Os01g07630.1 and Os01g59570.1) than their wild type siblings. Root specific growth phenotypes were noticed in only 1 line, which exhibited a decreased root length (Os03g16010.1). For 2 of these 6 genes (Os01g07630.1 and Os03g16010.1), we had 2 mutant lines analyzed per gene but the phenotype was only observed in one line. In these lines, the position and orientation of the T-DNAs added to the varietal background of these insertions may have impacted the phenotypes . Finally, we noticed that among the 6 genes with phenotypes on control medium, 3 (Os01g07630.1, Os01g59570.1, Os02g13410.1) were part of gene clusters. The Os01g07630.1 and Os01g59570.1 genes are part of clusters of two genes with Os01g07560.1 and Os01g59550.1, respectively. The mutant lines genotyped for these genes were not rearranged. The Os02g13410.1 gene belongs to cluster 2–5 with the Os02g13430.1 and Os02g13510.1 genes. In this cluster of three genes, a mutant line in Os02g13430.1 was also phenotyped but was not significantly affected in leaf 2 or root growth. This result could suggest that after duplication, these genes have perhaps diverged in their function.
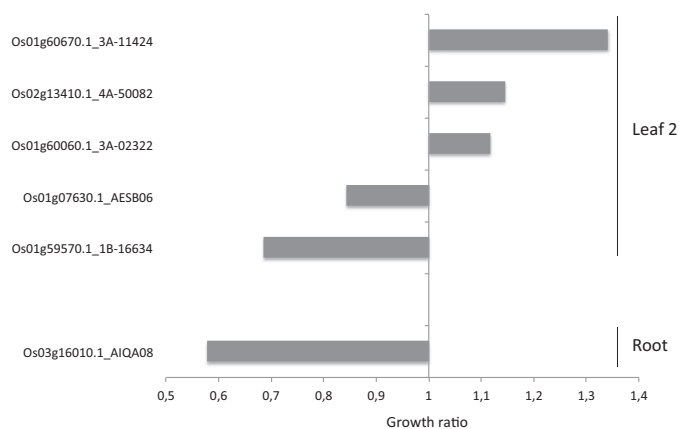
Fig. 3. Mutant lines affected in leaf 2 and root growth.
Ratio of mean growths of mutant and azygous control siblings for leaf 2 and root at day 6.
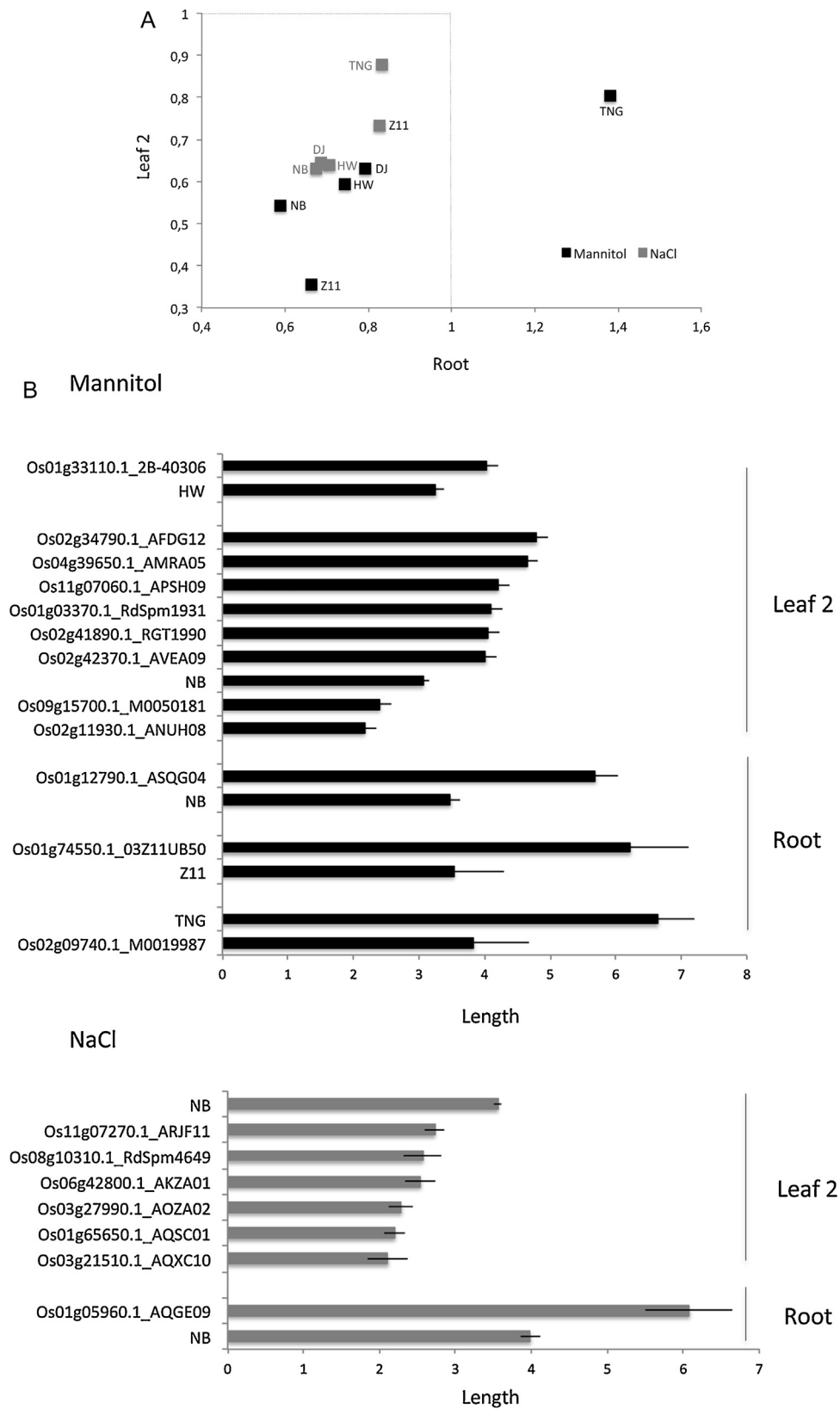
Fig. 4. Responses to mannitol and NaCl stresses.
(A) Responses of varietal controls (NB, HW, DJ, TNG, and Z11) to mannitol and NaCl stresses. Ratio of mean lengths of plants grown on stress medium vs. MS/2 control medium at day 6. (B–D) Insertion lines exhibiting more or less pronounced responses than their respective varietal controls to either mannitol or salt stresses in either leaf 2 or root growth (B), to both mannitol and salt stresses in either leaf2 or root growth (C), to either mannitol or salt stresses in both leaf 2 or root growths (D). (E) Insertion lines exhibiting different responses to mannitol and salt stresses in leaf 2 and root growths. Mannitol (black); NaCl (grey); lengths in cm.
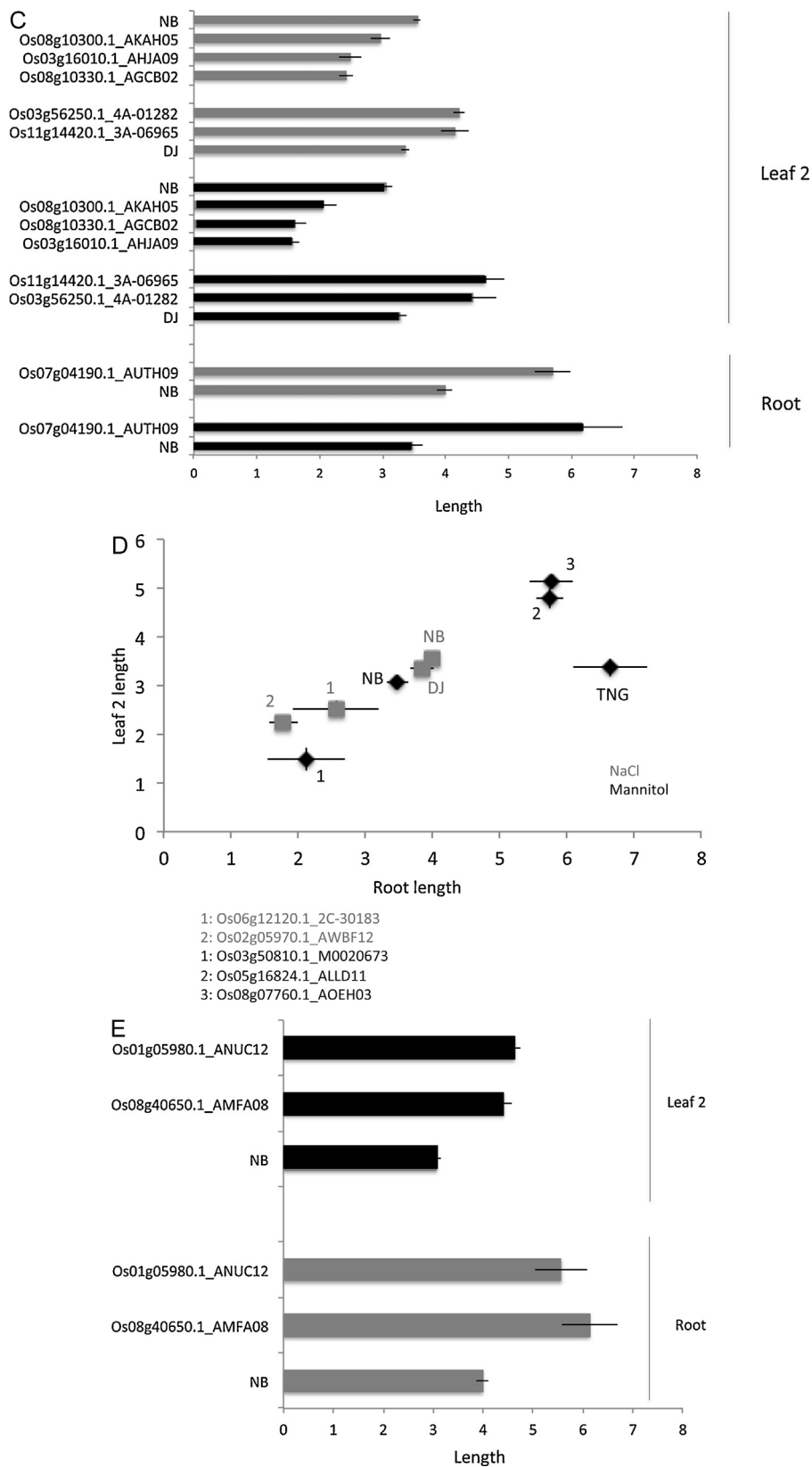
Fig. 4. (Continued).

3.4. Conditional phenotypes under abiotic stresses are observed in 32 mutant lines

For abiotic stress experiments, we first analyzed the phenotypes of wild type NB, DJ, HW, TNG, and Z11 plants. We grew these seedlings on MS/2 medium supplemented with mannitol (120 mM) or salt (NaCl 120 mM) in Petri dishes. We measured the leaf 2 and seminal root sizes 6 days after germination (Fig. 4A and Suppl. Fig. 3). Our results showed that NB, DJ and HW varieties behaved

Table 3
Phenotypes observed in control and stress screens.

				MS/2	Mannitol	NaCl	Best blast hit on TAIR 10 for Arabidopsis homolog
Os01g03370.1			RdSpm1931		+		AT4G29990.1
Os01g05960.1		CLUSTER_1-1	AQGE09			+	AT3G47570.1
Os01g05980.1			ANUC12		+	+	AT3G47570.1
Os01g07630.1	OsSERL5	CLUSTER_1-2	AESB06	−			AT1G60800.1 (AtNIK3)
Os01g12790.1			ASQG04		+		AT3G47570.1
Os01g33110.1		CLUSTER_1-3	2B-40306		+		AT4G08850.1
Os01g59570.1		CLUSTER_1-5	1B-16634	−			AT4G29990.1
Os01g60060.1			3A-02322	+			AT1G79620.1
Os01g60670.1			3A-11424	+			AT3G56370.1 (IRK)
Os01g65650.1			AQSC01			−	AT1G72180.1
Os01g74550.1			03Z11UB50		+		AT2G37050.1
Os02g05970.1		CLUSTER_2-1	AWBF12		−	−	AT1G72300.1
Os02g09740.1			M0019987		−		AT4G22130.1 (AtSRF8)
Os02g11930.1		CLUSTER_2-3	ANUH08		−		AT3G47570.1
Os02g13410.1		CLUSTER_2-5	4A-50082	+			AT5G25930.1
Os02g34790.1	XIK1	CLUSTER_2-6	AFDG12		+		AT4G08850.1
Os02g41890.1			RGT1990		+		AT2G02220.1 (AtPSKR1)
Os02g42370.1			AVEA09		+		AT3G47570.1
Os03g16010.1			AIQA08	−			AT1G31420.1 (AtFEI1)
			AHJA09		−	−	
Os03g21510.1			AQXC10			−	AT5G58300.1
Os03g27990.1			AOZA02			−	AT1G53730.1 (AtSRF6)
Os03g50810.1	OsTMK		M0020673		−		AT1G66150.1 (AtTMK1)
Os03g56250.1		CLUSTER_3-1	4A-01282		+	+	AT4G39270.1
Os04g39650.1			AMRA05		+		AT5G06940.1
Os05g16824.1		CLUSTER_5-1	ALLD11		+	+	AT1G56130.1
Os06g12120.1			2C-30183			−	AT2G13790.1 (AtSERK4)
Os06g42800.1			AKZA01			−	AT4G22130.1 (AtSRF8)
Os07g04190.1		CLUSTER_7-1	AUTH09		+	+	AT5G65700.1 (AtBAM1)
Os08g07760.1			AOEH03		+		AT1G34210.1 (AtSERK2)
Os08g10300.1		CLUSTER_8-1	AKAH05		−	−	AT1G56130.1
Os08g10310.1			RdSpm4649			−	AT1G56130.1
Os08g10330.1			AGCB02		−		AT1G56130.1
Os08g40650.1			AMFA08		+	+	AT4G29990.1
Os09g15700.1			M0050181		−		AT1G28440.1 (AtHSL1)
Os11g07060.1		CLUSTER_11-1	APSH09		+		AT3G47570.1
Os11g07270.1			ARJF11			−	AT3G47570.1
Os11g14420.1			3A-06965		+	+	AT5G48380.1 (AtBIR1)

approximately the same way on mannitol or NaCl medium, albeit with slight differences. Both leaf and root lengths were reduced under abiotic stresses compared to MS/2 medium in 30–50% and 20–40% respective ranges with variety specificities. Interestingly, in TNG plants, roots were longer on mannitol- but not on salt- supplemented medium whereas reduction of leaf length was comparable under NaCl and mannitol. For variety Z11, we noticed that leaf 2 size was much more affected on mannitol than on NaCl.

Keeping in mind these varietal specificities and in the aim of detecting conditional stress-responsive genes, we selected the 69 homozygous mutant lines (corresponding to 63 *LRR-RLK* genes) that did not exhibited a phenotype when grown on control MS/2 medium (Fig. 2 (Box 6)). We grew them under mannitol (120 mM) and salt (120 mM) stresses. We then compared the measurements obtained for leaf 2 and roots with their respective varietal controls grown under same stress conditions (Dunnett test, $p < 0.05$, Suppl. Table 5). First, we observed that 37 lines (53.6%) did not present a phenotype in either of the two stress conditions. For the lines showing differences compared to the varietal control, we analyzed separately leaf 2 and root phenotypes in each stress condition (Fig. 4B–D). Some lines exhibited phenotypes for a specific organ and under a particular stress (Fig. 4B). We scored 9 and 6 lines affected in leaf growth on mannitol or salt compared to their controls, respectively. Three lines were specifically affected in root growth on mannitol with 2 (Os01g12790.1 ASQG04 and Os01g75550.1 03Z11UB50) and 1 (Os02g09740.1 M0019987) exhibiting longer and shorter roots than their controls, respectively. On salt, we recorded only reduced growth of leaves, suggesting that these mutant lines were all more sensitive to salt. We

also observed one line (Os01g05960.1 AQGE09) with longer roots. Six lines presented a comparable phenotype on mannitol and NaCl media (Fig. 4C). Among these lines, 3 lines (Os08g10300.1 AKAH05, Os03g16010.1 AHJA09, Os08g10330.1 AGCB02) and 2 lines (Os03g56250.1 4A-01282, Os11g14420.1 3A-06965) exhibited reduced or enhanced root growth, respectively. Only one line exhibited longer roots on both media (Os07g04190.1 AUTH09). For gene Os03g16010.1, we have shown above that roots of line AIQA08 were shorter than control on MS/2 medium. Under abiotic stresses, another line tagging this gene (AHJA09) had shorter leaves. Our results also showed that 5 lines had phenotypes affecting both leaves and roots (Fig. 4D). For the 2 lines presenting these combined phenotypes on NaCl, plants were smaller than their respective controls (Os06g12120.1 2C-30183, Os02g05970.1 AWBF12). On mannitol, we recorded 1 line with smaller plants (Os03g50810.1 M0020673) and 2 lines with bigger plants (Os05g16824.1 ALLD11, Os08g07760.1 AOEH03). Interestingly, for 2 other lines analyzed (Os01g05980.1 ANUC12 and Os08g40650.1 AMFA08), we observed different although consistent phenotypes on mannitol and NaCl (Fig. 4E). Indeed, leaves were longer than control only on mannitol, and roots were longer only on NaCl medium. We also compared the results obtained in the different lines tagging the same gene. For example, 2 lines carried allelic inserts in gene Os03g21510.1 (AQXC10 and AHQF09). A leaf phenotype was observed only on salt medium in one of these lines. Among the 3 lines tagging Os03g27990.1 (ATDG06, AOZA02, and ARMB09) only leaves of AOZA02 plants had a reduced size compared to wild type on salt. All these results are summarized in Fig. 5.
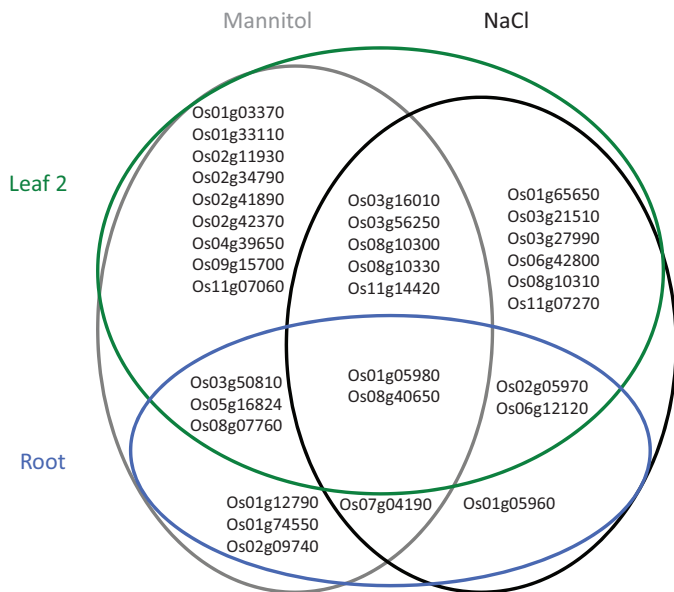
Fig. 5. Venn diagram of LRR-RLK genes putatively involved in conditional abiotic stress responses.

4. Conclusion

All together, these results show that the screen we have performed is a first step to establish a list of 37 *LRR-RLK* genes potentially involved in developmental and adaptive abiotic stress responses (Table 3). Among the genes with already described functions in rice, we highlighted a potential role for OsTMK and XIK1 in the response to mannitol (Table 1 and Table 3). Interestingly, this reverse genetics approach has already been performed on root-expressed *LRR-RLKs* in *Arabidopsis* [60]. We have compared our gene list with the one published in this study for abiotic stress responses. In both studies, the putative involvement of BAM1 in abiotic stress responses has been noticed.

Our results also show that unrelated mutations are segregating at high frequency in mutant line collections. In consequence, a careful analysis of sibling plants has to be done to try to eliminate most of the unrelated mutations. Despite our efforts to get rid of these extra mutations, some phenotypes described in our manuscript could be, at least in part, due to these additional mutations. Thus, fine functional analyses are also required to confirm the phenotypes observed for all these mutant lines. Nonetheless, our screen has been successful at identifying 37 *LRR-RLK* genes that are linked to growth phenotypes either under control or abiotic stress conditions. These lines will be further investigated through comprehensive functional analyses. Furthermore, our mutant collection is also available for other screens to investigate new LRR-RLK functions.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.plantsci.2015.06.019

References

[1] H.C. Godfray, et al., Food security: the challenge of feeding 9 billion people, Science 327 (2010) 812–818.

[2] Q. Zhang, Strategies for developing Green Super Rice, Proc. Natl. Acad. Sci. U. S. A. 104 (2007) 16402–16409.

[3] R.A. Wing, Q. Zhang, Perspective rice 2020 revised: an urgent call to mobilize and coordinate rice functional genomics research worldwide, in: Q. Zhang, R.A. Wing (Eds.), Genetics and Genomics of Rice, Springer, New York, 2013, pp. 387–390.

[4] International Rice Genome Sequencing Project, The map-based sequence of the rice genome, Nature 436 (2005) 793–800.

[5] J. Jacquemin, et al., The International Oryza Map Alignment Project: development of a genus-wide comparative genomics platform to help solve the 9 billion-people question, Curr. Opin. Plant Biol. 16 (2013) 147–156.

[6] 3,000 rice genomes project, The 3,000 rice genomes project, Gigascience 3 (2014) 7.

[7] S.A. Goff, et al., A draft sequence of the rice genome (*Oryza sativa* L. ssp. japonica), Science 296 (2002) 92–100.

[8] J. Yu, et al., A draft sequence of the rice genome (*Oryza sativa* L. ssp. indica), Science 296 (2002) 79–92.

[9] M. Wang, et al., The genome sequence of African rice (*Oryza glaberrima*) and evidence for independent domestication, Nat. Genet. 46 (2014) 982–988.

[10] Q.J. Zhang, et al., Rapid diversification of five *Oryza* AA genomes associated with rice adaptation, Proc. Natl. Acad. Sci. U. S. A. 111 (2014) E4954–E4962.

[11] F.-J. Wei, et al., International consortium of rice mutagenesis: resources and beyond, Rice 6 (2013) 39.

[12] G. Droc, et al., Mutant resources for functional analysis of the rice genome, in: Q. Zhang, R.A. Wing (Eds.), Genetics and Genomics of Rice, Springer, New York, 2013, pp. 81–115.

[13] A.L. Eamens, et al., A bidirectional gene trap construct suitable for T-DNA and Ds-mediated insertional mutagenesis in rice (*Oryza sativa* L.), Plant Biotechnol. J. 2 (2004) 367–380.

[14] A. Miyao, et al., Target site specificity of the Tos17 retrotransposon shows a preference for insertion within genes and against insertion in retrotransposon-rich regions of the genome, Plant Cell 15 (2003) 1771–1780.

[15] L.J. van Enckevort, et al., EU-OSTID: a collection of transposon insertional mutants for functional genomics in rice, Plant Mol. Biol. 59 (2005) 99–110.

[16] C. Sallaud, et al., High throughput T-DNA insertion mutagenesis in rice: a first step towards in silico reverse genetics, Plant J. 39 (2004) 450–464.

[17] D.H. Jeong, et al., T-DNA insertional mutagenesis for activation tagging in rice, Plant Physiol. 130 (2002) 1636–1644.

[18] C.M. Kim, et al., Rapid, large-scale generation of Ds transposant lines and analysis of the Ds insertion sites in rice, Plant J. 39 (2004) 252–263.

[19] C. Wu, et al., Development of enhancer trap lines for functional analysis of the rice genome, Plant J. 35 (2003) 418–427.

[20] Y.I. Hsing, et al., A rice gene activation/knockout mutant resource for high throughput functional genomics, Plant Mol. Biol. 63 (2007) 351–364.

[21] C.S. Kumar, et al., Efficient insertional mutagenesis in rice using the maize En/Spm elements, Plant J. 44 (2005) 879–892.

[22] Q. Yuan, et al., The TIGR rice genome annotation resource: annotating the rice genome and creating resources for plant biologists, Nucleic Acids Res. 31 (2003) 229–233.

[23] G. Droc, et al., OryGenesDB 2008 update: database interoperability for functional genomics of rice, Nucleic Acids Res. 37 (2009) D992–995.

[24] G. Droc, et al., OryGenesDB: a database for rice reverse genetics, Nucleic Acids Res. 34 (2006) D736–D740.

[25] Y. Kawahara, et al., Improvement of the *Oryza sativa* Nipponbare reference genome using next generation sequence and optical map data, Rice 6 (2013) 41.

[26] S.H. Shiu, et al., Comparative analysis of the receptor-like kinase family in *Arabidopsis* and rice, Plant Cell 16 (2004) 1220–1234.

[27] S.H. Shiu, A.B. Bleecker, Receptor-like kinases from *Arabidopsis* form a monophyletic gene family related to animal receptor kinases, Proc. Natl. Acad. Sci. U. S. A. 98 (2001) 10763–10768.

[28] A.B. Shiu, Plant receptor-like kinase gene family: diversity, function, and signaling, Sci. STKE 2001 (2001) RE22.

[29] L.A. Gish, S.E. Clark, The RLK/Pelle family of kinases, Plant J. 66 (2011) 117–127.

[30] M. Antolin-Llovera, et al., Knowing your friends and foes–plant receptor-like kinases as initiators of symbiosis or defence, New Phytol. 204 (2014) 791–802.

[31] B. Kemmerling, et al., A genome-wide survey for *Arabidopsis* leucine-rich repeat receptor kinases implicated in plant immunity, Front. Plant Sci. 2 (2011) 88.

[32] S.A. Morillo, F.E. Tax, Functional analysis of receptor-like kinases in monocots and dicots, Curr. Opin. Plant Biol. 9 (2006) 460–469.

[33] I. De Smet, et al., Receptor-like kinases shape the plant, Nat. Cell Biol. 11 (2009) 1166–1173.

[34] J. Li, J. Chory, A putative leucine-rich repeat receptor kinase involved in brassinosteroid signal transduction, Cell 90 (1997) 929–938.

[35] E.D. Shpak, Diverse roles of ERECTA family genes in plant development, J. Integr. Plant Biol. 55 (2013) 1238–1250.

[36] M. van Zanten, et al., The many functions of ERECTA, Trends Plant Sci. 14 (2009) 214–218.

[37] K.U. Torii, et al., The *Arabidopsis* ERECTA gene encodes a putative receptor protein kinase with extracellular leucine-rich repeats, Plant Cell 8 (1996) 735–746.

[38] S.E. Clark, et al., The CLAVATA1 gene encodes a putative receptor kinase that controls shoot and floral meristem size in *Arabidopsis*, Cell 89 (1997) 575–585.

[39] L. Gomez-Gomez, T. Boller, FLS2: an LRR receptor-like kinase involved in the perception of the bacterial elicitor flagellin in *Arabidopsis*, Mol. Cell 5 (2000) 1003–1011.

[40] C. Zipfel, et al., Perception of the bacterial PAMP EF-Tu by the receptor EFR restricts *Agrobacterium*-mediated transformation, Cell 125 (2006) 749–760.

[41] V. Hecht, et al., The *Arabidopsis* SOMATIC EMBRYOGENESIS RECEPTOR KINASE 1 gene is expressed in developing ovules and embryos and enhances embryogenic competence in culture, Plant Physiol. 127 (2001) 803–816.

[42] C. Albrecht, et al., The *Arabidopsis thaliana* SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASES1 and 2 control male sporogenesis, Plant Cell 17 (2005) 3337–3349.

[43] J. Colcombet, et al., *Arabidopsis* SOMATIC EMBRYOGENESIS RECEPTOR KINASES1 and 2 are essential for tapetum development and microspore maturation, Plant Cell 17 (2005) 3350–3361.

[44] K.H. Nam, J. Li, BRI1/BAK1, a receptor kinase pair mediating brassinosteroid signaling, Cell 110 (2002) 203–212.

[45] J. Li, et al., BAK1, an *Arabidopsis* LRR receptor-like protein kinase, interacts with BRI1 and modulates brassinosteroid signaling, Cell 110 (2002) 213–222.

[46] J. Li, Multi-tasking of somatic embryogenesis receptor-like protein kinases, Curr. Opin. Plant Biol. 13 (2010) 509–514.

[47] M.D. Lehti-Shiu, et al., Evolutionary history and stress regulation of plant receptor-like kinase/pelle genes, Plant Physiol. 150 (2009) 12–26.

[48] X. Sun, G. Wang, Genome-wide identification, characterization and phylogenetic analysis of the rice LRR-kinases, PLoS One 6 (2011) e16079.

[49] P.Y. Huang, L. Zimmerli, Enhancing crop innate immunity: new promising trends, Front. Plant Sci. 5 (2014) 624.

[50] M. Lorieux, et al., In-depth molecular and phenotypic characterization in a rice insertion line library facilitates gene identification through reverse and forward genetics approaches, Plant Biotechnol. J. 10 (2012) 555–568.

[51] D. Mieulet, et al., Reverse genetics in rice using Tos17, Methods Mol. Biol. 1057 (2013) 205–221.

[52] M. Lartaud, et al., PHIV-RootCell: a supervised image analysis tool for rice root anatomical parameter quantification, Front. Plant Sci. 5 (2015).

[53] A. Dievart, et al., Leucine-rich repeat receptor kinases are sporadically distributed in eukaryotic genomes, BMC Evol. Biol. 11 (2011) 367.

[54] S.R. Eddy, A new generation of homology search tools based on probabilistic inference, Genome Inform. 23 (2009) 205–211.

[55] K. Nonomura, et al., The MSP1 gene is necessary to restrict the number of cells entering into male and female sporogenesis and to initiate anther wall formation in rice, Plant Cell 15 (2003) 1728–1739.

[56] F. Sabot, et al., Transpositional landscape of the rice genome revealed by paired-end mapping of high-throughput re-sequencing data, Plant J. 66 (2011) 241–246.

[57] M. Nakano, R. Samejima, H. Iida, Retraction: Mechanosensitive channel candidate MCA2 is involved in touch-induced root responses in *Arabidopsis*, Front. Plant Sci. 6 (2015).

[58] A. Miyao, et al., Molecular spectrum of somaclonal variation in regenerated rice revealed by whole-genome sequencing, Plant Cell. Physiol. 53 (2012) 256–264.

[59] H. Stroud, et al., Plants regenerated from tissue culture contain stable epigenome changes in rice, Elife 2 (2013), e00354.

[60] C.A. ten Hove, et al., Probing the roles of LRR RLK genes in *Arabidopsis thaliana* roots using a custom T-DNA insertion set, Plant Mol. Biol. 76 (2011) 69–83.

[61] W.Y. Song, et al., A receptor kinase-like protein encoded by the rice disease resistance gene, Xa21, Science 270 (1995) 1804–1806.

[62] H. Hu, et al., A receptor like kinase gene with expressional responsiveness on *Xanthomonas oryzae* pv. *oryzae* is essential for Xa21-mediated disease resistance, Rice 8 (2015) 1.

[63] X. Sun, et al., Xa26, a gene conferring resistance to *Xanthomonas oryzae* pv. *oryzae* in rice, encodes an LRR receptor kinase-like protein, Plant J. 37 (2004) 517–527.

[64] Y. Xiang, et al., Xa3, conferring resistance for rice bacterial blight and encoding a receptor kinase-like protein, is the same as Xa26, Theor. Appl. Genet. 113 (2006) 1347–1355.

[65] R. Takai, et al., Analysis of flagellin perception mediated by flg22 receptor OsFLS2 in rice, Mol. Plant Microbe Interact. 21 (2008) 1635–1642.

[66] C. Yamamuro, et al., Loss of function of a rice brassinosteroid insensitive1 homolog prevents internode elongation and bending of the lamina joint, Plant Cell 12 (2000) 1591–1606.

[67] A. Nakamura, et al., The role of OsBRI1 and its homologous genes, OsBRL1 and OsBRL3, in rice, Plant Physiol. 140 (2006) 580–590.

[68] T. Suzaki, et al., The gene FLORAL ORGAN NUMBER1 regulates floral meristem size in rice and encodes a leucine-rich repeat receptor kinase orthologous to *Arabidopsis* CLAVATA1, Development 131 (2004) 5649–5657.

[69] X. Zhao, et al., OsTDL1A binds to the LRR domain of rice receptor kinase MSP1, and is required to limit sporocyte numbers, Plant J. 54 (2008) 375–387.

[70] H. Peng, et al., A putative leucine-rich repeat receptor kinase, OsBRR1, is involved in rice blast resistance, Planta 230 (2009) 377–385.

[71] E. van der Knaap, et al., Expression of a gibberellin-induced leucine-rich repeat receptor-like protein kinase in deepwater rice and its interaction with kinase-associated protein phosphatase, Plant Physiol. 120 (1999) 559–570.

[72] X. Chen, et al., An XA21-associated kinase (OsSERK2) regulates immunity mediated by the XA21 and XA3 immune receptors, Mol. Plant 7 (2014) 874–892.

[73] Y. Ito, et al., Expression of SERK family receptor-like protein kinase genes in rice, Biochim. Biophys. Acta 1730 (2005) 253–258.

[74] D. Li, et al., Engineering OsBAK1 gene as a molecular tool to improve rice architecture for high yield, Plant Biotechnol. J. 7 (2009) 791–806.

[75] B. Singla, et al., Structural characterization and expression analysis of the SERK/SERL gene family in rice (*Oryza sativa*), Int. J. Plant Genomics 2009 (2009) 539402.

[76] D. Song, et al., Molecular characterization and expression analysis of OsBISERK1, a gene encoding a leucine-rich repeat receptor-like kinase, during disease resistance responses in rice, Mol. Biol. Rep. 35 (2008) 275–283.

[77] J. Sakaguchi, et al., COE1, an LRR-RLK responsible for commissural vein pattern formation in rice, Plant J. 63 (2010) 405–416.

[78] C.F. Huang, et al., A rice mutant sensitive to Al toxicity is defective in the specification of root outer cell layers, Plant Cell Physiol. 50 (2009) 976–985.

[79] C.F. Huang, et al., A leucine-rich repeat receptor-like kinase gene is involved in the specification of outer cell layers in rice roots, Plant J. 69 (2012) 565–576.

[80] R. Thilmony, et al., The LP2 leucine-rich repeat receptor kinase gene promoter directs organ-specific, light-responsive expression in transgenic rice, Plant Biotechnol. J. 7 (2009) 867–882.

[81] F. Wu, et al., Plasma membrane receptor-like kinase leaf panicle 2 acts downstream of the DROUGHT AND SALT TOLERANCE transcription factor to regulate drought sensitivity in rice, J. Exp. Bot. 66 (2015) 271–281.

[82] S. Park, et al., Molecular dissection of the response of a rice leucine-rich repeat receptor-like kinase (LRR-RLK) gene to abiotic stresses, J. Plant Physiol. 171 (2014) 1645–1653.

[83] Y. Cheng, et al., New changes in the plasma-membrane-associated proteome of rice roots under salt stress, Proteomics 9 (2009) 3100–3114.

[84] Y. Zou, et al., OsRPK1, a novel leucine-rich repeat receptor-like kinase, negatively regulates polar auxin transport and root development in rice, Biochim. Biophys. Acta 1840 (2014) 1676–1685.

[85] Y.S. Law, et al., Molecular characterization and comparative sequence analysis of defense-related gene, *Oryza rufipogon* receptor-like protein kinase 1, Int. J. Mol. Sci. 13 (2012) 9343–9362.

[86] C.C. Shi, et al., Overexpression of the receptor-like protein kinase genes AtRPK1 and OsRPK1 reduces the salt tolerance of *Arabidopsis thaliana*, Plant Sci. 217–218 (2014) 63–70.

[87] S.Q. Ouyang, et al., Receptor-like kinase OsSIK1 improves drought and salt stress tolerance in rice (*Oryza sativa*) plants, Plant J. 62 (2015) 316–329.

[88] Y. Jiang, et al., XIAO is involved in the control of organ size by contributing to the regulation of signaling and homeostasis of brassinosteroids and cell cycling in rice, Plant J. 70 (2012) 398–408.

[89] C. Chen, et al., A two-locus interaction causes interspecific hybrid weakness in rice, Nat. Commun. 5 (2014) 3357.

[90] X. Zha, et al., Over-expression of the rice LRK1 gene improves quantitative yield components, Plant Biotechnol. J. 7 (2009) 611–620.