Multiplex mutagenesis of four clustered CrRLK1L with CRISPR/Cas9 exposes their growth regulatory roles in response to metal ions

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Resolving functions of closely linked genes is challenging or nearly impossible with classical genetic tools. Four members of the Catharanthus roseus receptor-like kinase 1-like (CrRLK1L) family are clustered on Arabidopsis chromosome five. To resolve the potentially redundant functions of this subclass of CrRLK1Ls named MEDOS1 to 4 (MDS1 to 4), we generated a single CRISPR/Cas9 transformation vector using a Golden Gate based cloning system to target all four genes simultaneously. We introduce single mutations within and deletions between MDS genes as well as knock-outs of the whole 11 kb gene cluster. The large MDS cluster deletion was inherited in up to 25% of plants lacking the CRISPR/Cas9 construct in the T2 generation. In contrast to described phenotypes of already characterized CrRLK1L mutants, quadruple mds knock-outs were fully fertile, developed normal root hairs and trichomes and responded to pharmacological inhibition of cellulose biosynthesis similar to wildtype. Recently, we demonstrated the role of four CrRLK1L in growth adaptation to metal ion stress. Here we show the involvement of MDS genes in response to Ni2+ during hypocotyl elongation and to Cd2+ and Zn2+ during root growth. Our finding supports the model of an organ specific network of positively and negatively acting CrRLK1Ls.

The ability to easily manipulate an organism's genome is highly desirable for both basic and applied plant science1. Diverse methods to genetically modify plants have revolutionized basic plant research as well as agriculture, e.g. engineering crop plants to increase yield, adapt to climate change, or to improve pathogen resistance. However, in contrast to many other organisms, targeted genome modification based on homologous recombination has proven very inefficient in higher plants2 making precise genome engineering difficult. With the advent of modern genome editing, especially with the development of the RNA-guided clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated protein 9 (Cas9)-based genome engineering system in 2012, targeted gene editing has now also become a reality in plants3. Multiple reports showed efficient and heritable CRISPR/Cas9-mediated genome engineering in a variety of plant species4–7.

The CRISPR/Cas9 system is able to target specific genomic regions through a guide RNA (gRNA), which is designed to contain a 20-nucleotide long region complementary to the DNA sequence of interest. Cas9 then introduces a site-specific double stranded DNA break (DSB), which is repaired by endogenous DNA repair mechanisms. Error-prone non-homologous end joining (NHEJ) machinery re-joins the broken DNA ends, often introducing small insertions or deletions (indels) at the cleavage site which can cause frameshift mutations or...
premature termination codons. A major advantage over other methods is the option to generate multiple simultaneous modifications simply by using more than one gRNA.

We adapted the GreenGate system, a Golden Gate based cloning system for plants, for the modular, customizable assembly of CRISPR/Cas9 constructs. This enables the rapid generation of constructs containing any combination of a variety of promoters, plant-codon optimized Cas9 variants (Cas9, Cas9n, dCas9), protein tags, selection markers, and single or multiple gRNA coding genes.

Here, we used this system to target a genetically uncharacterized cluster of four members of the *Catharanthus roseus* receptor-like kinase 1-like (*CrRLK1L*) gene family. The *CrRLK1L* gene family is characterized by a conserved cytoplasmic kinase domain and an extracellular region with one or two potential malectin-like sugar binding domains, which might associate with cell wall components in plants. It has recently been shown that FERONIA (FER) and ANXUR1 (ANX1) and ANXUR2 (ANX2) and BUDDHA’s PAPER SEAL1 and 2 (BUPS1 and BUPS2) are receptors for secreted peptides of the RAPID ALKALINIZATION FACTOR (RALF) family. To date, ten of the 17 members of the *CrRLK1L* gene family in *Arabidopsis thaliana* have been genetically investigated. The mutant phenotypes indicate their involvement in cell wall sensing during development and upon environmental stress. A subgroup of four highly homologous *CrRLK1L* members is clustered in an approximately 11 kb region on chromosome five. Similar to other members of the family named after Greek (*THESEUS1, HERKULES1* and 2) or Roman mythology (*FERONIA, ANXUR1* and 2), we named the four members of this *CrRLK1L* cluster *MEDOS1* to 4 (*MDS1* to 4) after the putative half-brother of THESEUS.

The genetic analysis of closely linked genes is difficult or even impossible if double, triple, and quadruple mutant combinations are needed to resolve their putative redundant functions. Our goal was to create both single and multiple gene knock-outs of the *MEDOS* family members through a multiplexing approach using CRISPR/Cas9 editing with a single plant transformation vector. By choosing three guide RNAs (gRNAs) which together target all four genes of the *MEDOS* cluster, we were not only able to create single nonsense mutations in each gene in the cluster, but also chimeras between gene cluster members, creating every possible truncation with a single CRISPR/Cas9 editing construct. We present an efficient screening strategy that enables the identification of edited plants lacking the CRISPR/Cas9 construct in the T2 generation. Phenotypic characterization of the *mds* mutants suggests their involvement in growth responses to increased metal ion concentration. Accordingly, *MDS* genes belong to a complex network of *CrRLK1L* s that both positively and negatively regulate growth.

**Results**

**The MEDOS gene cluster.** The 17 members of the *CrRLK1L* family share a basic structure, with one or two extracellular malectin-like domains at their N-terminus, a transmembrane domain, and an intracellular serine/threonine kinase domain at their C-terminus (Fig. 1a, Supplemental Fig. S1a,b). Sequence identity of the kinase domain varies between 28.2% and 95.1% and of the malectin-like domains between 16.7% and 87.6%.
Supplemental Fig. S1c,d). Phylogenetic tree analysis of full length proteins revealed that the MEDOS proteins form two subclasses with around 44% amino acid identity (Fig. 1b; Supplemental Fig. S1c). MDS1 (At5g38990)/MDS2 (At5g39000) share an overall amino acid identity of 76.9% while MDS3 (At5g39020) and MDS4 (At5g39030) only 66.7% (Supplemental Fig. S1c). The extracellular malectin-like containing domains of MDS1 and MDS2 are 70.5% and those of MDS3/MDS4 69.1% identical (Supplemental Fig. S1d). The kinase domains are 95.1% and 82.2% identical, respectively. Apart from the four MDS genes, this chromosomal region contains two small open reading frames. The reading frame of At5g39024 is 96 bp and At5g39010 is 542 bp. At5g39024 encodes a peptide of 31 amino acids with sequence homology to a conserved region in the kinase domain of MDS3 and MDS4 as well as At1g66930, At1g66920, At1g66910, At1g66980, At1g66990, At1g67000, and At5g38260. RNA-Seq data indicate that At5g39024 is expressed in leaves and seedlings (Supplemental Fig. S2). At5g39010 encodes a hypothetical 169 amino acid protein with unknown function and is expressed in seedlings, leaves, and flowers (Supplemental Fig. S2).

Design of the knock-out construct. To set up a flexible, modular CRISPR/Cas9 system, we cloned the promoter-, Cas9-, terminator-, gRNA scaffold-, and selection marker- sequences into GreenGate entry vectors. GreenGate entry vectors were combined into a single plant transformation vector by the Golden Gate reaction. We aimed to produce frameshift or nonsense mutations in each gene, and therefore chose gRNA sequences as close to the 5′ end as possible. Three gRNAs were chosen that target less homologous regions coding for the malectin-like domain at the 5′ end of the MDS genes (Supplemental Fig. S1a). Together, these three gRNAs are predicted to target all four MDS genes (Fig. 2a, Supplemental Fig. S1a). The final transformation vector contained the ubiquitin4–2 promoter from Petroselinum crispum, Arabidopsis codon optimized Cas9, the pea3A terminator from Pisum sativum, a glufosinate ammonium resistance gene (BASTA), and three gRNA modules each consisting of the Arabidopsis U6–26 promoter, a guide, and the gRNA scaffold (Fig. 2b).

Analysis of the T1 generation. Transformants were selected with BASTA. Genomic DNA was isolated from rosette leaves of 27 T1 plants and analyzed by PCR. As the largest deletion (MDS1 fused to MDS4) is easy to score, we designed primers that bind upstream of the gRNA1 targeting site and downstream of the gRNA3 binding site. 23 out of the 27 plants showed an approximately 11 kb deletion (Supplemental Fig. S3). Sequencing confirmed the identity of the deletion specific PCR product.

Analyses of the T2 and T3 generation. To determine the number of T-DNA insertions, putative fertility defects, and possible lethal phenotypes, the T2 progeny of the 27 transgenic plants were evaluated for their segregation behavior on BASTA (Supplemental Table S1). Most transgenic lines contained a single T-DNA insertion based on the segregation ratio on BASTA in the T2. The following lines were chosen to select BASTA sensitive, and thus T-DNA negative, individuals: line 2, 3, 4, 9, 11, 12, 13, 18, 22, and 26. From 718 T2 plants tested, 109 were both BASTA sensitive and negative for Cas9 by PCR, confirming the elimination of the transgene (Table 1). From these 109 plants, 17 (15.6%) possessed the large deletion (Table 1). The frequency of the large deletion varied in the progeny of primary transformants from 6.7% to 25% (Table 1). Furthermore, smaller deletions were also detected (Table 1). From these smaller deletions, the most frequent was found between MDS1 and MDS3 followed by deletions between MDS1 and MDS2, suggesting that gRNA1 was the most effective.

Figure 2. Schematic illustration of the four targeted MDS genes and the CRISPR/Cas9 T-DNA construct. (a) Open reading frames of the MDS gene cluster. Cas9 cutting sites are indicated with scissors. Blue and red arrows indicate primer binding sites for genotyping T1 plants. (b) The vector contains three U6–26-promoter driven gRNA modules targeting the four members of the MDS gene cluster. Cas9 is driven by the ubi4–2 promoter from parsley. LB, T-DNA left border; RB, T-DNA right border; pPCUBI4–2, ubiquitin4–2 promoter from Petroselinum crispum; CAS9, Arabidopsis codon optimized Cas9; tPea3(A), pea3A terminator from Pisum sativum; U6–26, Arabidopsis U6–26 (Pol-III promoter), gRNA1, gRNA2, gRNA3, gRNA/scaffold RNA modules; BASTA, glufosinate ammonium resistance gene.
We found one event where gRNA1 also edited combinations (Table 1, Supplemental Fig. S4a and b). Additionally, some large deletions were at unexpected sites MDS4 or MDS2, MDS3 and site onwards (Fig. 2a; fragment e; Fig. 3a,c and d). If only gRNA2 was active deletions with the potential to produce a fusion between MDS4 and MDS2, MDS3 and functions of the two additional short open reading frames in the MDS cluster, At5g39010 and At5g39024.

Table 1. Summary of the screening for BASTA sensitive CRISPR/Cas-9 edited T2 plants with deletions between MDS genes and mutations within MDS genes. # plants tested, show the numbers of plants used to examine BASTA sensitivity with the brushing method. % refers to the percentage of BASTA sensitive plants that contain the mds1-4 deletion. The Δ columns display the mutants with smaller deletions between the numbered MDS genes. The columns mds1, mds2, mds3, mds4 correspond to the number of mutant alleles within individual MDS genes.

| Line | # plants tested | BASTA sensitive | Δ mds 1-4 | Δ mds1-2 | Δ mds2-3 | Δ mds 3-4 | Δ mds1-3 | Δ mds2-4 | mds1 | mds2 | mds3 | mds4 |
|------|-----------------|-----------------|-----------|-----------|-----------|-----------|-----------|-----------|------|------|------|------|
| 2    | 19              | 7               | 0         | 0         | 0         | 0         | 0         | 0         | 0    | 0    | 0    | 0    |
| 3    | 120             | 20              | 5         | 1         | 0         | 1         | 5         | 0         | 3    | 6    | 1    | 3    |
| 4    | 13              | 4               | 1         | 25.0      | 0         | 1         | 5         | 0         | 3    | 6    | 1    | 3    |
| 8    | 20              | 3               | 0         | 0         | 0         | 0         | 0         | 0         | 0    | 0    | 0    | 0    |
| 9    | 65              | 5               | 0         | 0         | 0         | 0         | 0         | 0         | 0    | 0    | 0    | 0    |
| 11   | 108             | 15              | 1         | 6.7       | 1         | 0         | 0         | 2         | 0    | 6    | 7    | 6    |
| 12   | 119             | 9               | 2         | 22.2      | 1         | 1         | 0         | 0         | 1    | 4    | 1    | 0    |
| 13   | 100             | 16              | 4         | 25.0      | 0         | 0         | 0         | 0         | 2    | 7    | 2    | 1    |
| 18   | 15              | 3               | 0         | 0         | 0         | 0         | 0         | 0         | 0    | 0    | 0    | 0    |
| 22   | 119             | 24              | 4         | 16.7      | 1         | 0         | 1         | 1         | 0    | 2    | 9    | 3    |
| 26   | 20              | 3               | 0         | 0         | 0         | 0         | 0         | 0         | 0    | 0    | 0    | 0    |
| Sum  | 718             | 109             | 17        | 15.6      | 6         | 1         | 3         | 8         | 1    | 17   | 33   | 13   |

In addition to deletion of the entire cluster as well as deletions between only two or three MDS genes, mutations within the genes themselves were detected (Table 1). We identified mutant lines with mutations in one, two, three, and all four MDS genes. In particular, the quadruple MDS mutant served as a control to test for putative functions of the two additional short open reading frames in the MDS cluster, At5g39010 and At5g39024.

To identify homozygous lines, plants from the T3 generation were genotyped with either deletion PCRs or T7 endonuclease assays and sequencing. All deletion lines segregated in a Mendelian manner for a single locus. Since the mutations in the MDS genes were either heterozygous or biallelic, the T3 segregation analyses allowed us to determine whether the alleles in the different MDS genes are coupled or in the repulsion phase.

**Mutation spectrum of the CRISPR/Cas9 edited MDS gene cluster.** Since the gRNAs were positioned in the coding region, the mutations often caused a frameshift, resulting in a non-sense mutation with a premature stop codon (Fig. 3; Supplemental Fig. S4). The activity of gRNA1 and gRNA3 resulted in the large MDS1 to 4 deletions with the potential to produce a fusion between MDS1 and MDS4 with frameshifts from the gRNA target site onwards (Fig. 2a; fragment e; Fig. 3a,c and d mds4(KG)). Only the large deletion in mutant mds12NN resulted in an in-frame fusion with the potential to express a chimeric protein with one malec tin-like domain from MDS1 and two from MDS4 (Fig. 3a,c and d). The activity of gRNA1 and gRNA2 produced deletion mutants between MDS1 and MDS2 or MDS3 (Fig. 2a; fragments a and c; Supplemental Fig. S4b). If only gRNA2 was active deletions between MDS2 and MDS3 occurred (Fig. 2a; fragment b). Deletion products of gRNA2 and gRNA3 were between MDS2 or MDS3 and MDS4 (Fig. 2a; fragments d and f). Mutant lines were identified with all of these possible combinations (Table 1, Supplemental Fig. S4a and b). Additionally, some large deletions were at unexpected sites (Supplemental Fig. S3c). We found one event where gRNA1 also edited MDS4 (mds4(125)) which has CTG instead of the canonical PAM motif NGG (Supplemental Fig. S4c). Another incident is the mutant mds12ab with a MDS2–4 fusion event (Supplemental Fig. S4c). Although the MDS2–4 fusion is positioned around gRNA3, this event is most likely due to failed repair after a gRNA2 guided cut. In mutant mds13NN, a 561 bp deletion occurred after the target site of gRNA2 and in mutant mds13, a single adenine was inserted at the correct editing site of gRNA2 in MDS2, but the fusion between MDS2 and MDS3 happened roughly 400 bp downstream (Supplemental Fig. S1a; Supplemental Fig. S4c). In mutant mds13NN, the MDS1 target site is fused with At5g39024. Additionally, mds312NN contains a 546 bp deletion in MDS4 which starts close to target site of gRNA3 and ends close to an off target site of gRNA1 (Supplemental Fig. S4c). This rearrangement probably has no consequences since the resulting gene fusion has a very early stop codon.

By using T7 endonuclease assays and sequencing, mutations within the MDS genes were identified where only one (mds4(125)), two (mds113M, mds14T, mds26O), three (mds14aZ, mds14h, mds14h), or all four (mds14aZ, mds14h, mds14h, mds14h) MDS genes were mutated (Fig. 3b and c; Supplemental Fig. S4d). In the case of mds14aZ, the mutation in MDS1 does not result in a frameshift but rather a 15 amino acids deletion at the N-terminal end of the second malec tin-like domain, which is highly conserved throughout all CrRLK1Ls (Fig. 3d). In contrast, in mds14aZ, the mutation in MDS4 leads to a two amino acid deletion without a frameshift in a less conserved N-terminal region of the first malec tin-like domain. In contrast, in mds14aZ, the mutation in MDS4 leads to a two amino acid deletion with a highly conserved arginine (R) of the first malec tin-like domain (Supplemental Fig. S1b). A similar case is for mds14ab and mds14ab. Where in mds14ab all MDS genes except MDS4 are mutated, in mds14ab the highly conserve arginine (R) of MDS4 is deleted and a amino acid substitution threonin 26 to isoleucine (26F) and a two amino acid deletion happened in MDS3 (Supplemental Fig S1b and S5a and b). These mutants enable the evaluation of gene specific contributions to the potentially redundant functions of the MDS genes and the potential redundant functions of MDS3 and MDS4 (Fig. 3d).
Expression of the MDS genes. In reverse genetic approaches, expression analysis of uncharacterized genes indicates where and under which conditions these genes might be crucial. Quantitative gene expression evaluations are therefore a prerequisite for subsequent functional analysis. Although many microarray data are available, MDS1 (At5g38990) and MDS2 (At5g39000) share an oligo on the Affymetrix Arabidopsis microarray (ID 249480_s_at) and thus it was not possible to determine the individual contribution of MDS1 and MDS2 in microarray databases such as the Botany Array Resource (BAR)31–33 and Genevestigator34,35. Our RT-qPCR revealed that MDS1 is the most highly expressed gene of the cluster in all organs tested followed by MDS4 and MDS3 (Fig. 4, Supplemental Figs S2 and S6). MDS2 had the lowest expression (Fig. 4 insert, Supplemental Fig S6).

In contrast to MDS1, MDS3, and MDS4, which are most highly expressed in rosette and cauline leaves, MDS2 is most strongly expressed in seedlings and roots (Fig. 4, Supplemental Fig S6). Our expression data are consistent with that of the 113 RNA-Seq deposited in the ThaleMine/Araport 36,37 database (Supplemental Fig S2).

As expected, the expression of MDS1, MDS2, and MDS3 in the mds1–4 deletion mutants was absent while the 3’ end of the MDS4 gene was detectable (Supplemental Fig S7). As indicated above, the large deletion in the

Figure 3. Characterization of the CRISPR/Cas9 edited mutants mds4GG, mds12NN, mds11Z1, and mds11Z3. Sequences of mutants with (a) deletions between MDS1 and 4 and (b) small mutations within individual MDS genes. gRNAs are marked by colored boxes and sequences, protospacer adjacent motif (PAM) motifs are indicated in purple. Underlined letters indicate the reading frames. (c) Schematic representation of the mutations. Numbers indicate deleted or inserted bases. (d) Domain structure of the putative truncated and chimeric proteins. Black bars represent deletions.
mds4GG mutant leads to a frameshift and premature stop codon while mds12NN has an in-frame fusion and potentially expresses a chimeric protein (Fig. 3d). In the mutants mds11aZ1 and mds11aZ3, all MDS genes were expressed. However, due to the frameshifts and premature stop codons, it is likely that the mRNAs are either degraded by nonsense-mediated decay (NMD) or truncated proteins are synthesized (Supplemental Fig. S7).

Phenotypic analyses of the mds1-4 deletion mutants. Mutant phenotypes are essential for the identification of the in vivo role of a gene product at the cellular or organismal levels. To date, the function of eight CrRLK1L family members have been genetically investigated, revealing a wide range of biological roles all related to growth.

Of these ten, six are involved in fertilization. While fer mutant ovules are defective in pollen tube reception21, BUPS1/2 and ANX1/2 are required to maintain pollen tube integrity17,19,22,38. Recently, it was shown that ERU is involved in Ca²⁺ dependent pollen tube growth and eru pollen are less competitive than wildtype pollen in fertilization39. Based on these gametophytic phenotypes, we expected abnormal segregation ratios of the mds1-4 deletion mutants. However, all mds1-4 deletion mutants are recessive allele (Table 2).

A mutant in CURVY1 was identified due to its defective trichome and pavement cell morphogenesis18. Since most of the MDS genes are strongly expressed in leaves, we analyzed trichome patterning and morphogenesis. Neither trichome morphology nor density was altered in comparison to wildtype (Fig. 5a and b). Additional CrRLK1L mutant phenotypes have been published for instance for larger seeds and nearly no root hairs in fer mutants25, while eru mutants develop short root hairs40. Neither root hair phenotypes nor altered seed size or total seed weight were detectable in the mds mutants (Fig. 5c–f).

We then searched for phenotypes associated with gain- and loss-of-function mutants of the cell wall integrity sensor THESEUS1 (THE1). THE1 was identified in a screen for suppressors of the short hypocotyl and ectopic lignification phenotypes of cellulose biosynthesis mutants and interpreted to be a negative regulator of growth upon cellulose biosynthesis defects41. The loss-of-function allele the1–6 is less sensitive to pharmacological inhibition of cellulose synthesis by isoxaben with regards to hypocotyl elongation and deposition of ectopic lignin42,43 (Supplemental Fig. S8). Neither hypocotyl elongation nor ectopic lignification phenotypes were detectable in mds mutants, suggesting that the MDS genes are not negative regulators of growth upon cellulose biosynthesis inhibition.

MDS genes function in growth responses to heavy metals and trace elements. We recently recognized four CrRLK1L family members to be involved in growth adaptation to heavy metals and trace elements29. Based on these findings, root growth and etiolated hypocotyl elongation were evaluated in the mds1-4 deletion mutants. Similar to29, etiolated hypocotyl lengths were quantified on 1/10 Hoaglands medium supplemented with either Ni²⁺, Cd²⁺, Cu²⁺, Zn²⁺, or Pb²⁺ salts. On control media the different mds mutants developed the same hypocotyl length. However, mds mutants where all genes were either deleted or mutated (mds4GG, mds12NN, mds11aZ1, mds11aZ3, mds12NN)

| Allele    | total | mutant [obs./exp.] | heterozygote [obs./exp.] | wildtype [obs./exp.] | Chi-Square | p-value |
|-----------|-------|--------------------|--------------------------|---------------------|------------|---------|
| mds4GG    | 98    | 23 [24.5]          | 48 [49]                 | 27 [24.5]           | 0.3673     | 0.83    |
| mds12NN   | 83    | 21 [18.25]         | 38 [36.5]               | 24 [18.25]          | 2.2877     | 0.32    |

Table 2. Segregation ratio analyses of the large deletion alleles, mds4GG and mds12NN. obs. is the abbreviation of observed and exp. for expected genotypes.

Figure 4. Expression of the four different MDS genes in different organs. Shown are the means and standard errors (SEM) of RT-qPCR data of three biological (for cauline leaves two) and three technical replicates, normalized to the reference gene AP2M. Abbreviations in the insert are s for seedlings, r for rosette leaves, c for cauline leaves, and f for flowers.
mds11aZ1, mds3d15) were significantly shorter on 20 µM and 30 µM of Ni2+ (Fig. 6a, Supplemental Fig. S5c). In contrast, mds11AZ3, the allele where MDS3 is wildtype and MDS4 lacks only two amino acids at the beginning of the first malectin-like domain performed similar to wildtype (Fig. 6a and b). Similarly behaved the mds3d7 seedlings where MDS4 is wildtype and MDS3 has a nonsense mutation (Supplemental Fig. S5c). These results indicate that at least the MDS3 and MDS4 act redundantly since both have to be mutated to cause a growth phenotype on Ni2+.

This growth behavior is opposite to mutants of THE1 (the1), HERKULES 1 (herk1) and 2 (herk2), and similar to THE1 gain-of-function alleles and the fer-4 mutant 29. Hypocotyl elongation of the mds mutants was similar to wildtype on all other metal ions (Fig. 6).

Figure 5. Various developmental phenotypes of wildtype and the mds1-4 large deletion mutants. (a) Rosette leaves and close-ups of their adaxial surfaces, (b) average +/- SEM trichome density on the adaxial surface of rosette leaves of ten individuals in one experiment, (c) root hairs of 5 days old seedlings cultivated on MS medium supplemented with 2.5% sucrose and 1.5% agar, (d) imbibed seeds, (e) average total seed weight +/- SEM of 10 individual plants, (f) average seed size +/- SEM of at least 33 seeds of two to three seed stocks for wildtype, mds4GG and mds12NN, and of one seed stock for fer-4. Star indicates significant difference according to Student's t-test with *p < 0.05 to wildtype.
When quantifying root growth on these metal ions a different picture emerged. First, root growth was faster on control media, significantly for the mds12NN large deletion allele (Fig. 7a). We therefore normalized our data to control conditions (Fig. 7b) which revealed that both mds deletion alleles were less sensitive to Cd$^{2+}$ and hypersensitive to Zn$^{2+}$ (Fig. 7b). Cd$^{2+}$ tolerance has not yet been observed for CrRLK1L mutants as herk1, herk2, and the1 roots are shorter on Cd$^{2+}$ containing medium compared to wildtype. The significantly reduced root growth on Zn$^{2+}$ of the mds deletion alleles is similar to herk1, the1–6 and fer–4. Taken together, our data suggest that the MDS genes have functional redundancies and are similar to THE1, HERK1, HERK2, and FER involved in growth adaptation upon exposure to metal ions. Therefore, the MDS genes contribute to the complex network of CrRLK1Ls that positively and negatively affect growth.

**Discussion**

Members of gene families in plants are often highly redundant and must be simultaneously mutated for functional analysis. Complicated crossing schemes have been the method of choice provided single mutants were available. When the family members are localized in a cluster, their mutation was an extremely difficult task, but indispensable for genetic analysis. Using the CRISPR/Cas9 system, it has become easy to knock-out several genes simultaneously by either using one gRNA targeting homologous regions in all genes or by using several gRNAs targeting each gene individually (or a combination of both approaches). Furthermore, it is now possible to delete whole gene clusters or large chromosomal regions. To develop a flexible cloning system, we used GreenGate as it is a very versatile system. One simply needs to engineer gRNA modules for each genome editing target and combine it with appropriate promoters, Cas9 versions, and terminators for the goal of the specific project. Compared to Gateway-based vector systems, which produce large “scars” due to their recombination sequences, the GreenGate system uses only 4 bp overhangs which can easily be designed to produce “scar-less” constructs.

As with all vector-based CRISPR/Cas9 systems, it is necessary to select plants that have lost the construct to avoid somatic effects due to continuing activity of Cas9. We decided on BASTA-sensitivity assays by brush application to single rosette leaves in the T2 generation to select CRISPR/Cas9 edited plants that lack the original T-DNA insertion. In our opinion, this selection is superior to the use of EGFP and DsRed fluorescence in the...
seed coat as no tedious seed selection under a fluorescent microscope is needed. Additionally, false negatives due to poor expressed fluorescing reporters are reduced. Furthermore, brushing with BASTA can be repeated if the phenotype is not indisputable. By brushing only one leaf the rest of the plant will survive and can be used to screen for the mutations generated by CRISPR/Cas9. This method is certainly superior to antibiotic selection which does not allow for negative selection. To account for the possibility that an additional truncated CRISPR/Cas9 construct without the BASTA gene was inserted into the genome we also performed PCR to confirm the absence of the CAS9 gene. While the larger deletions were easily scored with PCR and agarose gel electrophoresis, care must be taken for the accurate detection of single nucleotide polymorphisms (SNPs) and small indels. Fast prescreening for of these mutations was done with the T7 endonuclease and CEL1 assays. While both mismatch-specific endonuclease cleavage assays provided accurate detection of single extrahelical nucleotides of indels, they were less effective at detecting single base substitutions. These specificities have already been published in other detailed surveys, demonstrating that T7 endonuclease preferentially identified insertions and deletions, whereas CEL1 (commercially available under Surveyor nuclease S) was better for recognizing substitutions. A recent, direct comparison showed that the T7 endonuclease assay was extremely sensitive in detecting indels, with it being possible to detect one heterozygous individual out of a pool of eight samples. The CEL1/Surveyor nuclease was again found to be better at detecting single-nucleotide substitutions, but the specificity and efficiency was lower due to nonspecific cleavage products. In conclusion, all mismatch-specific enzyme assays underestimate CRISPR/Cas9 editing events and sequencing the target region after PCR amplification is highly recommended.

From the phenotypic analyses of the CRISPR/Cas9 generated mds mutants it is clear that they are not involved in signaling cascades similar to their characterized GrRLK1L family members during unchallenged development. Furthermore, the mds1-4 large deletion mutants did not exhibit phenotypes associated with either loss-of-function or gain-of-function alleles of the cell wall integrity sensor THE1 under cellulosic deficient conditions. However, based on the growth assays on increased concentrations of diverse metal ions, the MDS genes appear to be involved in mediating adaptation to metal ion stress, a function which has been recently attributed to the GrRLK1L family members HERK1 and 2, THE1, and FER. Similar to mutants of FER, THE1, HERK1 and 2, hypocotyl elongation was also differently affected compared to root growth in the mds1-4 deletion mutants. Furthermore, the MDS genes act redundantly, since shorter etiolated hypocotyls on Ni²⁺ were only significant if all four MDS genes were either deleted or mutated. The mds1-4 deletion mutants developed longer roots on Cd²⁺, a phenotype which has not been previously observed, while the response to Zn²⁺ was similar to mutants of THE1, HERK1 and FER. Thus, the MDS mutants add to the complex picture of the GrRLK1L family as important players in a complex network of signaling cascades positively and negatively regulating growth and

![Figure 7](image_url)
cell elongation. Why different metal ions trigger specific CrRLK1Ls, and how they stimulate opposite growth effects, remains to be solved in the future. It is possible that the extracellular malectin–like domains directly bind metal ions since many carbohydrate binding lectins and carbohydrate–binding modules (CBM) need at least one metal ion for structural stabilization15 or even require metal ions such as calcium to oligomerize and bind carbohydrate ligands16,24. On the other hand, peptide ligands such as the FER specific RALF15 and RALF2316, or the pollen–specific BUPS1/2-ANX1/2 receptor heteromer which bind RALF4, RALF19, and RALF3417 might bind metal ions. Mature RALF peptides have four highly conserved cysteines60 which might bind metal ions and are often involved in redox sensing and therefore in metal perception and differentiation, albeit indirectly61,62. The third possibility relates to the putative carbohydrate binding feature of the extracellular malectin–like domain. For ANX1 and ANX2, Boisson-Dernier et al.63 proposed possible interactions with homogalacturonans; these pectin derived cell wall compounds or their degradation products are known to bind metal ions64. Wolf and Höfte65 propose a feedback loop between RALF and pectin modifying enzymes where RALF induced alkalinization of the cell wall would activate pectin–methylesterases (PMEs). PME activity would expose demethylesterified sugar acids, which are able to complex metal ions which changes the flexibility of the cell wall.

The involvement of the MDS genes in adaptation to heavy metal and trace element ions might not be their sole function since. The MDS genes, and in particular MDS3, was strongly induced upon UV-B exposure in the publicly deposited microarray database (Supplemental Fig. S9). Salt stress and high osmolarity oppositely affected expression in roots and shoots and extreme temperatures generally reduced expression (Supplemental Fig. S9). Recently, it was shown that FER, THE1 and ANXUR1/2 are involved in immune responses to biotic infections46,68,69,70. Looking at the microarray expression data, the MDS genes, particularly MDS3, were strongly induced upon bacterial infection or bacterial and oomycete elicitor treatments, while fungal infections also induced MDS4 (Supplemental Fig. S10a). Thus, our genome edited mutants provide an attractive genetic resource to dissect the importance of the MDS genes in pathogen infections. Further, the mds mutants will support functional genetic analyses of the CrRLK1L gene family with the goal of understanding the molecular basis of their specificities, the identification of their ligands and putative interactions and their downstream signaling networks.

Experimental Procedures

**Plant lines and growth conditions.** For transformation, we used wild-type Arabidopsis thaliana accession Columbia (Col-0). Plants were grown at 22 °C with a 16-h light/8-h dark cycle.

**Expression analysis by quantitative real-time PCR.** Total RNA of snap–frozen seedlings grown on MS2.5 supplemented with 1% (w/v) agar (Duchefa), rosette leaves, cauline leaves, or flowers was isolated using a MS2.5 supplemented with 1% (w/v) agar (Duchefa), rosette leaves, cauline leaves, or flowers was isolated using a LiCl/CTAB method. After grinding roughly 100 mg frozen tissue, 1 mL of pre-heated RNA extraction buffer (2% [w/v] hexadecyltrimethylammonium bromide, CTAB; 2% [w/v] polyvinylpyrrolidone, PVP; 100 mM Tris/HCl pH 8.0; 25 mM EDTA; 2 M NaCl; 0.5 g/L spermidine and 2.7% [v/v] 2-mercaptoethanol) was added, mixed and incubated at 65 °C for 5 min. CTAB was removed through two extractions with 1 mL of ice-cold chloroform: isoamylalcohol (24:1) and centrifugation at 4 °C. RNA in the supernatant was precipitated with 250 μL 10 M LiCl at 4 °C for more than 1.5 h. After centrifugation and ethanol washes the pellet was dissolved in 20 μL RNAse free water and stored at −80 °C. RNA was quantified with Qubit (Invitrogen) and NanoDrop (Peqlab) systems. cDNA was synthesized from 2.5 μg RNA after RNase free DNase I digestion (Fermentas) with the M-MuLV H PLUS reverse transcriptase (Peqlab) as described72.

RT-qPCR expression analysis was performed using the Hot FirePol EvaGreen qPCR Mastermix (Solis Biodyne) with a Rotorgene 3000 (Qiagen). Primers for MDS1, MDS2, MDS3, and MDS4 as well as the reference genes UBIQUITIN EXTENSION PROTEIN 5 (UBQ5), TUBULIN9 (TUB9), and ADAPTOR PROTEIN-2 MU-ADAPTIN (AP2M) are listed in Supplemental Table S2. Absolute and relative expression was calculated with a dilution series of purified PCR fragments of known molar concentration in each RT-qPCR run. Each sample was measured in triplicate from 2–3 independent cDNAs. Amplon identity was verified by melting curve analysis.

**Sequence and in silico transcriptome analysis of MEDOS genes and proteins.** For multiple sequence analysis, all Arabidopsis CrRLK1L gene and protein sequences were aligned with the help of the MegAlign tool of the DNAStar (Lasergene) sequence analysis software. GeneDoc software was used for editing multiple sequence alignments73. Definition and sizing of the metallic domains was calculated by ClustalW. The tree was constructed using the Maximum Likelihood method with 1000 bootstrap repeats in MEGA6. Public transcriptomics data were consulted using the eFP browser31,75,76. Plant lines and growth conditions.

**Construction of vectors.** Promoter (ubiquitin–4–2 promoter from Petroselinum crispum), Cas9 (codon-optimized for Arabidopsis thaliana), terminator (pea3A terminator from Pisum sativum) and customizable sgRNA (driven by the Arabidopsis U6–26 promoter) sequences from H. Puchta66 were transferred to GreenGate entry vectors (Addgene #1000000036) using Sequence- and Ligation-Independent Cloning (SLIC)77. Primers (no. 1–10) are listed in Supplemental Table S2.

The guide RNA (gRNA) sequences targeting the MDSOS family members were selected with the tool at the Broad institute (www.broadinstitute.org/rnai/public/analysis-tools/sgrna-design), examined for specificity using CRISPR-P (http://crispr.hzau.edu.cn/CRISPR/) and inserted into the guide RNA scaffold (sgRNA) GreenGate entry vectors. At their 5’ end, the gRNAs additionally contained a reverse complement sequence of the following overhangs: forward primer: attg, reverse primer: aac (no. 11 to 16; Supplemental Table S2). After annealing, the primers were ligated into the entry vector that had been linearized with BpiI (New England Biolabs, FD1014). We used two entry vectors each with a complete gRNA module (promoter, customized gRNA, and scaffold).
insert additional gRNAs, gRNA modules were amplified by PCR and inserted into the destination vector with the Golden Gate reaction. To this end, PCR primers were designed with following overhangs: E (forward primer) and F (reverse primer) for gRNA2 and E (forward primer) and P (reverse primer) for gRNA3 (primers no. 17–20 Supplemental Table S2). For the Golden Gate reaction, 150 ng of the destination vector (pGGZ003) and 250 ng of each entry vector containing the promoter, Cas9, terminator, gRNA1, and BASTA resistance were mixed with 250 ng each of the gRNA2 and gRNA3 PCR products, 0.2 μL BSA protein (New England Biolabs, B90005), 2 μL ligase buffer, 1.2 μL T4 DNA ligase (ThermoFisher, TLM01), 1 μL BsaI (NEB, R0535S) and distilled water (to 20 μL). The reaction was incubated for 5 min at 30 °C followed by 5 min at 16 °C and repeated 50 times. Finally, the reaction was incubated for 30 min at 30 °C, for 5 min at 50 °C, and for 5 min at 80 °C. To eliminate not fully-ligated intermediate products, we added 0.85 μL ATP (25 mM) and 1 μL Plasmid-Safe ATP-dependent DNase (Epicentre, E3101K) and incubated for 60 min at 37 °C and for 30 min at 70 °C. Half of the reaction was transformed into chemically competent E. coli cells (NEB10beta strain) and selected on LB plates containing 100 μg/mL spectinomycin.

**Generation of stable transgenic Arabidopsis plants.** Agrobacterium tumefaciens GV3101 pSOUP+ cells were transformed with our plasmid using the freeze/thaw protocol. Agrobacterium-mediated Arabidopsis transformation was performed using the floral dip method. Transgenic plants were grown on soil and selected with glufosinate ammonium (BASTA) by spraying every three to four days with a solution of 20 μg/L BASTA three or four times.

**Mutant screening and sequence analysis T1 generation.** A small leaf of each T1 plant was used for genomic DNA extraction. Fragments surrounding the target sites were amplified by PCR using primers At5g38990-F and At5g39030-R (no. 21 and 22; Supplemental Table S2) and the Phusion Flash High-Fidelity PCR Master Mix (ThermoFisher Scientific, F-548). The presence of the largest truncation was tested via agarose gel electrophoresis. The PCR products were sequenced with the Sanger method using primer At5g38990 seqR (no. 23; Supplemental Table S2) and compared to wildtype sequences using ApE- A plasmid Editor.

**Analysis of the T2 and the T3.** To determine the number of T-DNA insertions, putative fertility defects, and possible lethal phenotypes, the T2 progeny of the 27 transgenic lines were plated on MS medium supplemented with 1% sucrose and 5 mg/L glufosinate (BASTA). After one week cultivation at 22 °C and continuous light the segregation ratio of BASTA resistant and sensitive seedlings per line was quantified (Supplemental Table S1). To isolate T2 plants lacking the T-DNA, seedlings were transferred from plates without BASTA to soil and one rosette leave was treated with a 100 mg/L BASTA solution containing 0.03% Silwet L77. Leaves of BASTA sensitive plants start to perish after two to three days. BASTA sensitive plants were additionally tested for BASTA resistance by spraying every three to four days with a solution of 20 mg/L BASTA three or four times. To test for the presence of the T-DNA in T3 plants, seedlings were transferred from plates without BASTA to soil and one rosette leave was treated with a 100 mg/L BASTA solution containing 0.03% Silwet L77. Leaves of BASTA sensitive plants start to perish after two to three days. BASTA sensitive plants were additionally tested for BASTA resistance by spraying every three to four days with a solution of 20 mg/L BASTA three or four times.

**Growth assays and phenotypic analyses.** Seeds were surface-sterilized in 5% (v/v) sodium hypochlorite, 0.5% Tween-20 (v/v) solution, rinsed three times with sterile deionized water, and transferred to plates with nutrient agar containing 1/10 strength Hoagland salts, 1% (w/v) sucrose, and 1% (w/v) agar (Duchefa). For metal ion treatments, sterile salt solutions were added after autoclaving to achieve final concentrations of 10 μM CdCl₂, 5 μM CuSO₄, 15–30 μM NiSO₄, 100 μM Pb(NO₃)₂, and 100 μM ZnSO₄. After two days of imbibition at 4 °C in the dark, plates were vertically incubated in a growth chamber at 22 °C with constant light (80 μmol.m⁻².s⁻¹). For measurements of etiolated hypocotyls, plates were wrapped in aluminum foil after exposure to light for five hours. The plates were scanned on day five after germination (dag) for root growth and hypocotyl measurements. The lengths were evaluated with ImageJ software by freehand tracking. Isoxaben treatments, lignin staining and seed size measurements are presented in supplemental materials and methods. Statistical evaluation and notched boxplots were calculated and drawn with Excel and the R software.

**Seed size measurement with ImageJ.** Seed size was measured in ImageJ. Pictures were binarised after setting the threshold in the Lab color space for b values to 129–255. The particles were then identified and the area measured automatically with the ‘Analyze Particles’ function. Two to three different seed stocks were used for msd deletion mutants and one for fer-4 as control.

The datasets generated during and analyzed during the current study are available from the corresponding author on reasonable request.

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Acknowledgements

The authors are grateful to Julian Kelner and Daniela Keck for their help in genotyping. We are thankful to Holger Puchta for the pPcUBI4 and U6–26 promoters, Arabidopsis codon optimized Cas9, the tPEA3(A) terminator and the gRNA promoter/scaffold. Thanks also to Lilian Nehlin which initially helped with the establishment of general CRISPR/Cas9 entry vectors. We thank also the anonymous reviewers for their careful reading of our manuscript and their valuable comments and suggestions. The project was supported by a grant of the Austrian Science Fund and the French National Research Agency project ANR-FWF I 1725-B16 to MTH.

Author Contributions

J.R., J.M.W., V.S., M-T.H. conceived and designed the experiments; J.R., V.S., P.S. M.B. J.N. M.B. performed the experiments. J.R., P.S. and M.-T.H. analyzed the data; V.S., J.M.W., J.R. and M.-T.H. wrote the manuscript. P.S.-B. managed the cloning and transformation. All authors reviewed and approved the manuscript.

Additional Information

Supplementary information accompanies this paper at https://doi.org/10.1038/s41598-018-30711-3.

Competing Interests: The authors declare no competing interests.

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