Introduction

High quality and productive crops are often susceptible to a multitude of different pathogens and pests. Disease resistant crop varieties are commonly bred by the introgression of resistance (**R**) genes derived from wild crop relatives. Such **R**-genes often mediate recognition of race or isolate-specific effector proteins of the pathogen and the subsequent activation of defense responses in the plant. However, the frequent appearance of ‘new’ pathogen races in terms of their effector protein arsenal, renders many introgressed **R**-genes quickly ineffective, initiating a new cycle of **R**-gene discovery and introgression by breeders. Disease resistance breeding approaches that could overcome this lack of durability would therefore be highly advantageous.

Disease resistance can be obtained via several means. EMS mutagenesis approaches have regularly been used in Arabidopsis to obtain mutations in wild relatives and the subsequent introgression of these genes by mutagenesis. As resistance is not mediated by race-specific recognition of pathogens but by the absence of certain host genes, the **S**-gene approach is expected to be more durable. This is exemplified by powdery mildew resistant barley **mlo** lines, which have been successfully cultivated in agriculture for over 25 years [10].

Mutant screens which were performed later and aimed at dissecting the genetic basis of disease susceptibility, identified so called loss-of-susceptibility mutants showing disease resistance in absence of severe fitness costs [6–8]. This, together with recent insights in effector triggered susceptibility by manipulation of host factors led to a proposed new breeding strategy [9]. Instead of identifying **R**-genes in wild relatives and the subsequent introgression of these genes into elite cultivars, one could inactivate so called ‘Susceptibility’ (**S**) genes by mutagenesis. As resistance is not mediated by race-specific recognition of pathogens but by the absence of certain host genes, the **S**-gene approach is expected to be more durable. This is exemplified by powdery mildew resistant barley **mlo** lines, which have been successfully cultivated in agriculture for over 25 years [10].

**mlo** based powdery mildew susceptibility is conserved across plant species as impairment of barley **Mlo** orthologs in Arabidopsis [11], tomato [12] and pea [13–14] all result in resistance to adapted powdery mildews. For other **S**-genes it is unclear to which degree functions are conserved across plant families and whether impairment of such genes have potential breeding value. Here we report the functional characterization of tomato (*Solanum lycopersicum*) orthologs of the Arabidopsis **PMR4** and **DMR1** genes with respect to their **S**-gene function. **Powdery Mildew Resistant (PMR)** 4 was originally identified in a mutant screen for loss of susceptibility to *Erysiphe cichoracearum* [6]. **PMR4** encodes a callose synthase required for callose deposition in papillae. Resistance in *pmr4*
mutants, however, was found associated with salicylic acid accumulation rather than with the absence of callose deposition [15]. **Downy Mildew Resistance (DMR)** 1 was identified in a mutant screen for loss of susceptibility to **Hyaloperonospora arabidopsidis** [7]. Several different dmr1 alleles were identified, each mediating a significant level of resistance to H. arabidopsidis. **DMR1** encodes a homoserine kinase catalyzing the phosphorylation of homoserine to O-phospho-homoserine. Mutant plants accumulate homoserine and exogenous application of homoserine induces resistance in wild-type plants [16–17]. We observed reduced growth and proliferation of **Oidium neolycopersici** (tomato powdery mildew) in Arabidopsis dmr1 and pmr4 mutants as well as tomato plants in which expression of SIdMR1 and SIPMR4 was silenced, demonstrating that these S-gene functions are conserved between the two plant species. Resistance to **O. neolycopersici** is associated with severe fitness cost for SIdMR1 silencing but not for SIPMR4 silencing, indicating the latter has potential value for powdery mildew resistance breeding in tomato.

**Materials and Methods**

**Plant Growth and Cultivation**

**Arabidopsis thaliana** mutants **pmr4** in Col-0 background and **dmr1-1** to **dmr1-6** in Ler ed1-2 background are described in [6,7]. Plants were grown in plastic pots in potting soil (Horticoop, Lentse potgrond). Arabidopsis plants were grown in a growth chamber at 21°C and 19°C during the 8 h day and 16 h night periods respectively, a relative humidity of 70% and a light intensity of about 400 μmol m⁻² s⁻¹. Tomato plants (*S. lycopersicum* cv **Moneymaker**) were grown in greenhouses at 21°C and 19°C during the 16 h day and 8 h night periods respectively. Relative humidity was around 70% and light intensity was supplemented with 100 W/m² when light intensity dropped below 150 W/m².

**Pathogen Inoculations**

The Wageningen isolate of **Oidium neolycopersici** On-Ne [24] was maintained on tomato cv **Moneymaker** (MM) plants. Spore suspensions were obtained by washing heavily infected MM leaves in water. For disease assays, Arabidopsis and tomato plants of about 4 weeks old were sprayed with an inoculum of between 5–10⁵ spores per mL respectively. Fungal growth was evaluated at 8–14 days post inoculation (dpi). A disease index (DI) was used where 0 = no sporulation; 1 = slight sporulation, but about 30% foliar area affected; 2 = moderate sporulation, 30% to 60% foliar area affected; 3 = abundant sporulation, more than 60% area less than 5% foliar area affected; 4 = heavy sporulation, more than 60% area.

**Generation of Silencing Lines**

For generating the **SIPMR4** and **SIdMR1** silencing constructs we ordered **pUC57** clones containing a DNA fragment identical to the first 101 bp of the predicted coding sequence of Solyc07g053980 (**SIPMR4** h1) or a fragment identical to the first 140 bp of the predicted 3’ UTR of Solyc07g053980 (**SIdMR1**) flanked by attL sites from the Genescript (USA) company. Sequences are provided in Figure S1 in File S1. DNA fragments present in pUC57 were recombined into pHellsgate 8 [26] using the LR clonase enzyme mix from Invtrogen and transformed to chemical competent E. coli TOP 10 cells (Invitrogen). Constructs were subsequently extracted using the Plasmid extracting kit from Qiagen, sequenced to confirm the presence of the intended inserts and transformed to Agrobacterium strain AGL4:virG. For tomato transformation Moneymaker (MM) seeds were sterilized by incubation in 1% NaOCl for 20 minutes. Afterwards seeds were rinsed with water and sown on GEM medium (2.2 g MS salts/L, 10 g sucrose/L and 8 g Daishin agar/L; pH 5.8). Cotyledons from emerging plants were cut in several pieces and incubated for 10–15 minutes in an Agrobacterium suspension carrying the silencing constructs (OD600 of 0.125) in MSO (4.3 g MS salts/L, 0.4 mg thiamine/L, 100 mg Myo-inositol/L and 30 g sucrose/L; pH 5.6) plus 200 μM acetylsperine. Then the cotyledon pieces were blotted on sterile filter paper and placed with abaxial side down on GCF10 medium (4.3 g MS salts/L, 108.7 mg Vitamins Nitsch/L, 30 g sucrose/L, 8 g agar/L, 1.5 mg Zeatine riboside/L and 0.2 mg IAA/L; pH 5.8) plus 200 μM acetylsperine. This was then incubated in the dark for 48 hours at 25°C before transferring the explants to plates containing GCF10 medium plus 300 mg/L timentin and 100 mg/L kanamycin and subsequent incubation at 25°C in the light. After 3 weeks, the medium was refreshed. After 6–8 weeks emerging calli were excised from explants and transferred to GCF11 medium (4.3 g MS salts/L, 108.7 mg Vitamins Nitsch/L, 30 g sucrose/L, 8 g agar/L and 1.9 mg Zeatine riboside/L; pH 5.8) plus 300 μg/L timentin and 100 mg/L kanamycin. Emerging shoots were transferred to MS30B5 medium (4.3 g MS salts/L, 112 mg Vitamin B5/L, 30 g sucrose/L, 8 g agar/L and 1.9 mg Zeatine riboside/L; pH 5.8) plus 100 mg/L kanamycin. When proper roots had developed transformants were brought to the greenhouse. They were transferred to plastic pots containing potting soil, and kept under a plastic cover for a few days to achieve higher humidity. After adaptation the plastic cover was removed. In total, eight RNAi::SIPMR4 and twelve RNAi::SIdMR1 primary transformants (T1) were grown in the greenhouse. After gene expression analysis (see below) for each silencing construct three T1 plants that showed the highest level of silencing were selected, and allowed to set seed (T2). For SIPMR4 silenced plants, from each T1 plant 16–20 T2 progeny was grown in the greenhouse, together with 19 MM plants as control, and used in a disease assay.

**Nucleic Acid Extraction and Q-PCR**

To determine presence of the silencing construct DNA was isolated from leaf material using the HotSHOT method [27]. A PCR was performed using NPTII-specific primers NPT3 (TCCGGCTATGACTGGGCACAAGA) and NPT4 (AA-GAAGGCCGATAAAGGCGATGCC) [28]. For gene expression analysis, 8–14 days after inoculation of tomato plants with **O. neolycopersici** the 3rd or 4th leaf was collected from each T1 or T2 transformant or MM control plant. Total RNA was extracted using the MagMAX-96 total RNA Isolation kit (Ambion) or the RNAeasy kit from Qiagen (Germany). RNA was treated with RNase-free DNase (Qiagen). The quantity of RNA was measured using a spectrophotometer and 250 ng-1 μg was used to synthesize
cDNA using the iScript cDNA synthesis kit (Bio-Rad). Relative transcript levels were determined in two technical replicates using the iQ SYBR Green supermix (Bio-Rad) and the CFX96 Real-Time system (Bio-Rad). PCR was performed with 10 ng cDNA using an annealing temperature of 60°C, and 40 cycles. To check for DNA contamination samples that had not been treated with reverse transcriptase were included as a control. Tomato EF1α (Solyc06g005060) transcript levels were used for normalisation by the \( \Delta \Delta C_t \) method. Primers used for determining relative transcript levels were: Fw- EF1α- ATTGGAAACGGATATGCCCCT; Rv- EF1α- TCCTTACCTGAACGCCTGTCA; Fw- SlPMR4- GCCGGCGGCGAGACAAGTTT; Rv- SlPMR4- CAGCGCCAGCCAGCCAGCCAGCCAAGCAAGCA; Fw- SlDMR1- TGGGGAGAATGGTGGAGGCG; Rv- SlDMR1- ACTCGTTTGGAATTGAGGCATAGTTGA. Primers to determine expression of the NPTII gene were: Fw-NPTII- ACTGGGCACAACAGACAATC and Rv-NPTII- TCGTCCTGCAGTTCATTCAG.

Disease severity was measured by Q-PCR quantification of \( O. \) neolycopersici biomass using the same samples as used for gene expression analysis. PCR was performed using primer pair Fw-On-CGCCAAAGACCTAACCCTAAAAC and Rv-On-AGGCCAAAGATCCGTTTGTGA, designed on ITS sequences specific to \( O. \) neolycopersici (GenBank accession number EU047564). The Fw-EF1α and Rv-EF1α primer pair was used as reference to normalize the plant DNA proportion by the \( \Delta \Delta C_t \) method.

Exogenous Application of Amino-acid Solutions

Amino acids L-homoserine, L-isoleucine, L-leucine, L-threonine and L-valine were obtained from Sigma-Aldrich. 5 mM solutions were sprayed onto leaves of four-weeks old tomato plants. The next day plants were inoculated with \( O. \) neolycopersici. Three days after inoculation the plants were sprayed again with the respective amino acid solution.

Statistical Analysis

For comparisons of means analysis of variance was performed. When significant differences were found (\( p<0.05 \)) a Tukey post hoc test was carried out to detect which means were different.

Results

Arabidopsis pmr4 Mutants are Resistant to \( O. \) neolycopersici

Previously \( PMR4 \) was identified as a locus conferring susceptibility to the powdery mildew \( E. \) cichoracearum in Arabidopsis [6,15]. As Arabidopsis can be a host for the tomato powdery mildew \( O. \) neolycopersici [18] we assessed the possible requirement of \( PMR4 \) for this pathogen. Inoculated \( pmr4 \) mutants showed reduced levels of fungal sporulation as compared to Col-0 wild-type plants (Figure 1A and B), indicating that Arabidopsis \( PMR4 \) is a functional \( S \)-gene for \( O. \) neolycopersici.

Powdery Mildew Resistance by Impairment of \( PMR4 \) is Conserved between Arabidopsis and Tomato in Absence of Severe Fitness Costs

To explore the possible conservation of \( PMR4 \) among different plant species for susceptibility to powdery mildews, putative tomato orthologs of Arabidopsis \( PMR4 \) were identified by searching the SOL Genomics Network (SGN) database for similar sequences. Two genes annotated as \( Solyc07g053980 \) (\( SIPMR4_h1 \)) and \( Solyc02g078230 \) (\( SIPMR4_h2 \)) were found, encoding proteins having higher sequence identity at the amino acid level with
For statistically significant. For among T1 progenies of all 3 families, but differences were not families 2 and 3, but not 4. Stem diameters were somewhat lower was observed for progenies carrying a reduction (although significant as indicated by asterisks) in plant height of 12 week old plants respectively. A slight is a functional ortholog of SlPMR4 (family 2, 3 and 4). T2 progenies harboring a silencing construct correlated with the presence of relatively low or high levels of SPMR4 transcripts respectively (Figure 2C and Figure S2 in File S1). T2 families were inoculated with O. neolycopersici and their level of susceptibility determined by quantification of the relative ratio between fungal and plant DNA at 8 dpi. Fungal growth was significantly less among silenced plants compared to non-silenced plants demonstrating SPMR4 is a functional S-gene ortholog of Arabidopsis PMR4 (Figure 2D). Resistant plants did not show any apparent reduction in size or altered leaf morphology as compared to susceptible progeny at the age of five weeks when inoculations were performed (Figure 2A and B). However, T2 progeny of family 2 and 3 harbouring a silencing construct showed slightly reduced growth after 12 weeks in the greenhouse compared to plants not harbouring a silencing construct (Figure 2E). In contrast, no significant difference in plant height was found for progeny of family 4 grown under the same conditions as progenies of families 2 and 3 (Figure 2F). Moreover, stem diameter at 12 weeks was on average reduced in the SlPMR4-silenced plants compared to the non-silenced progenies of all 3 families, although this difference was not statistically significant (Figure 2F). Together the data suggests that O. neolycopersici resistance by impairment of SPMR4 function can be achieved without severe growth reduction.

Impairment of DMR1 is Associated with O. neolycopersici Resistance

Previously, Arabidopsis dmri-1 mutants were shown to be resistant to the downy mildew H. arabidopsidis but not to the powdery mildew Golovinomyces orontii or the bacterial pathogen Pseudomonas syringae, which suggested DMR1 mediates susceptibility specifically to downy mildew [7]. However, we observed a significant reduction of tomato powdery mildew O. neolycopersici sporulation at 14 dpi on dmri-1-1, dmri-1-2 and dmri-1-5 plants as compared to the parental line Ler-eds1-2 (Figure 3A–G), suggesting that DMRI1 is a functional S-gene for tomato powdery mildew in Arabidopsis. All dmri1 mutants except dmri1-3 showed reduced growth compared to the parental line as evidenced by the lower amount of fresh weight recorded 5 weeks after sowing (Figure 3H), suggesting that impairment of DMRI1 has fitness costs. Reduction in growth was lowest in dmri1-3 and dmri1-6, the same lines that did not show resistance to O. neolycopersici. This suggests that the
weaker dm1 alleles do not effectively mediate resistance to tomato powdery mildew, in contrast to H. arabidopsidis [7].

Pathogen Resistance by Impairment of DMR1 is Conserved between Arabidopsis and Tomato

Using the AdDMR1 amino acid sequence as a query the SOL Genomics Network (SGN) database was searched for similar sequences. A single tomato gene annotated as Solyc04g008760 was identified encoding a putative homoserine kinase having 71% sequence identity with AdDMR1. A silencing construct was generated specifically targeting this gene, hereafter referred to as SlDMR1, and used to transform tomato MM plants. The relative transcript level of SlDMR1 was determined in 12 independent transformants. We only obtained T1 plants showing either less than 2-fold silencing of SlDMR1 or over 4-fold silencing. Plants showing over 4-fold silencing were of reduced stature, had light green leaves (Figure 4C) and in addition, produced no offspring. Progeny of T1 plants showing less than 2 fold silencing of SlDMR1 did not show reduced growth of O. neolycopersici (data not shown).

Discussion

The potential of knocking-down S-genes for durable disease resistance breeding in crops has largely been unexplored due to anticipated fitness costs of impaired S-gene alleles. However, identification of pathogen resistant mutants with no or minor associated fitness costs, together with recent insights in pathogen effector-triggered susceptibility by manipulation of host factors has brought [renewed] interest to this subject [9]. Here we tested if the S-gene function of PMR4 and DMR1 is conserved between Arabidopsis and tomato and explored the potential use of the tomato orthologs for breeding resistance to tomato powdery mildew.

We showed that Arabidopsis mutants harboring mutations in PMR4 or DMR1 are resistant to O. neolycopersici in addition to E. cichoracearum and H. arabidopsidis respectively, as was previously described [6–7]. Silencing of the tomato orthologs SlPMR4 and SlDMR1 resulted in O. neolycopersici resistance in tomato, indicating that their S-gene functions are conserved across plant species. Interestingly, no severe fitness costs in terms of reduced growth were found associated with SlPMR4 silencing (Figure 2A, B, E and F) making this gene a promising target for mutagenesis to obtain suitable Slpmr4 alleles for disease resistance breeding in tomato.
Powdery Mildew is Less Sensitive to dmr1-mediated and Homoserine-induced Resistance than Downy Mildew

All Arabidopsis dmr1 mutants were identified in a screen for loss of susceptibility to the oomycete *H. arabidopsidis* and were shown to be resistant to this pathogen, including dmr1-3 [16]. No significant reduction in plant growth was observed for this mutant (Figure 3H), indicating that *H. arabidopsidis* resistance can be obtained by impairment of DMR1 in absence of severe fitness costs. This might be different for dmr1-mediated resistance to the ascomycete *O. neolycopersici*, as dmr1-3 (and dmr1-6) did not show a significant reduction in fungal growth (Figure 3G). Moreover, *O. neolycopersici* resistance in tomato was only observed for RNAi lines in which transcript levels were over four-fold reduced (Figure 4A,B). There was, however, a severe negative effect in the strongly silenced S IDM1 lines as they showed growth retardation and did not produce offspring.

Arabidopsis dmr1 mutants accumulate homoserine and exogenous application of homoserine induces pathogen resistance [16–17]. Of all characterized dmr1 alleles, dmr1-3 encodes a homoserine kinase with the highest residual enzyme activity and homoserine accumulation was less in dmr1-3 mutants compared to other dmr1 mutants [16]. As dmr1-3 is resistant to *H. arabidopsidis* but not to *O. neolycopersici* it suggests that the first is more sensitive to homoserine accumulation in the host than the latter.

**Figure 4. Tomato S IDM1 is a S-gene for the tomato powdery mildew Oidium neolycopersici.** (A), relative transcript abundance of S IDM1 in multiple independent cuttings of untransformed Moneymaker (MM) plants or T1 transformants harbouring an RNAi::S IDM1 construct (Line2, 3 and 5). (B), Quantification of fungal growth at 8 days post inoculation (dpi). S IDM1 silenced plants support significantly less fungal growth compared to untransformed plants. (C), Leaves of an untransformed MM and S IDM1 silenced plant at 8 dpi. Silencing of S IDM1 results in reduced leaf size and yellowish colour. For A and B, Data indicate the mean of 3 or more biological replicates with error bars representing the standard error. Number of asterisks indicate degree of significance (**p<0.001**).

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**Mechanism of dmr1 and pmr4 Mediated Resistance**

The mechanism by which impairment of DMR1 mediates resistance is unclear. Experiments to test direct toxicity showed that *H. arabidopsidis* spore germination and germ tube growth is not inhibited by homoserine [16]. Impairment of DMR1 more generally perturbs amino acid homeostasis as dmr1 mutants show changes in their levels of the homoserine-derived amino acids threonine, isoleucine and methionine [16]. Perturbation of amino acid homeostasis was also observed in *rpm1* and *rpm2* mutants that accumulate lysine, threonine, methionine and isoleucine and are resistant to *H. arabidopsidis* but not the powdery mildew *G. orontii* [17]. In the study of Stuttmann et al. [17], exogenous application of threonine appeared to be a more potent inducer of *H. arabidopsidis* resistance than homoserine, in contrast to what was found by Van Damme et al. [16] and what we observed in tomato (Figure 5A). However, Stuttmann et al. [17] hypothesized that plants and oomycetes biosynthesize lysine via the diaminopimelate (DAP) pathway, whereas ascomycetes use the a-aminoadipate (AAA) pathway. Differences in interference of these biosynthetic pathways in the pathogen by amino acid perturbations in the host (or exogenously applied) might underlie the relative vulnerability of downy mildew (oomycete) as compared to powdery mildew (ascomycete).
There is no evidence for the activation of plant defence responses in the dmrl mutants or in homoserine-treated plants that are resistant to H. arabidopsidis. The SA-dependent defence marker gene PR-1 was not induced in dmrl mutants, nor was homoserine-induced resistance impaired in a large collection of Arabidopsis defence-signalling mutants [16]. In contrast, the Arabidopsis pmr4 mutants show constitutive activation of SA-dependent defences [15]. Resistance in pmr4 mutants was lost in double mutants in which the SA pathway is impaired [13]. This result indicated that SA accumulation rather than lack of pathogen-induced PMR4 callose synthase activity is the cause of resistance in pmr4 mutants to adapted powdery mildews. Recently, it was shown that overexpression of PMR4 resulted in elevated early callose deposition, leading to complete penetration resistance to both nonadapted and adapted powdery mildews. Further, overexpression of PMR4 did not influence SA and jasmonate-related pathways [29]. Therefore, PMR4 not only has an enzymatic function (callose synthase activity) but also plays a regulatory role (such as a negative regulator of SA pathway).

We expect that the resistance found in our SPMR4- and SIDMR1-silenced lines is due to the regulatory role of the PMR4 gene on defence-related pathways. As to the resistance shown in SIDMR1-silenced tomato lines, we expect that the direct toxicity of homoserine may be the cause, although we cannot exclude the possibility that the activation of plant defence responses may play a role. Further experiments are ongoing to monitor the fungal growth, callose deposition and marker genes for defence pathways in SPMR4- and SIDMR1-silenced lines. In addition, these RNAi lines are being crossed with tomato mutants defective in SA, jasmonic acid and ethylene pathways to verify whether the impairment of certain pathways compromises the resistance resulting from the disruption of SPMR4 or SIDMR1. The obtained results will clarify whether different mechanisms and/or cross-talk between the defence-related pathways are associated with the silencing of SPMR4 or SIDMR1.

Perspective for Breeding

Making use of impaired S-gene alleles to obtain durable disease resistance was recently proposed as a new breeding strategy [9]. Valuable S-gene alleles mediating a high level of resistance with a minimum of associated fitness costs are expected to be rare. However, state-of-the-art mutagenesis techniques [19–23] allow the creation and or detection of such rare alleles once S-genes have been identified in a particular crop species. Here we have shown that the S-gene functions of DMR1 and PMR4 are conserved between Arabidopsis and tomato, and therefore expected to be functional in other plant species as well. Silencing of SPMR4 did not result in severe fitness costs (reduced growth) suggesting that loss-of-function or hypomorphic alleles could mediate powdery mildew resistance with a minimum of fitness costs in tomato. SIDMR1 silencing was strongly associated with severe fitness costs in tomato and sldmr1 alleles conferring a high level of resistance in absence of severe effects on growth (and yield) might therefore be more difficult to obtain. However, the existence of the Arabidopsis dmr1-3 mutant that is resistant to downy mildew [7] without severe fitness costs (Figure 3H) suggests that similar alleles can be identified in at least some plant species. Identification of new S-gene alleles in model and crop species will contribute to future disease resistance breeding.

For many crop species genome sequences are or will soon become available, which will facilitate the identification of orthologs of S-genes in other plant species. Assuming that their function in disease susceptibility is conserved among different plant species, as is the case for MLO [11–14], PMR4 and DMR1, desired mutations can easily be obtained by (targeted) mutagenesis approaches [19–23] and applied in breeding crops with durable resistance. Thus this study presents a proof-of-concept research on the potential exploitation of orthologs of Arabidopsis S-genes in resistance breeding in crop species.

Supporting Information

File S1 Sequences of silencing fragments used to specifically silence tomato genes Solyc07g053980 (SPMR4) and Solyc04g006760 (SIDMR1), and experimental data showing the absence of cross-silencing of another tomato PMR4 family member Solyc02g078230 (SPMR4_h2) by the SPMR4_h1 silencing construct. (DOCX)

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Author Contributions

Conceived and designed the experiments: RpH YB. Performed the experiments: RpH AehmL DG. Analyzed the data: RpH AehmL DG YB. Contributed reagents/materials/analysis tools: RpH AehmL DG. Wrote the paper: RpH GvdA RgV YB.
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