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To cite this version:

Daniel I. Pacurar, Monica L. Pacurar, Andrea M. Pacurar, Laurent Gutierrez, Catherine Bellini. A novel viable allele of Arabidopsis [i]CULLIN1[/i] identified in a screen for [i]superroot2[/i] suppressors by next generation sequencing-assisted mapping. PLoS ONE, Public Library of Science, 2014, 9 (6), 8 p. 10.1371/journal.pone.0100846 : hal-01204145

HAL Id: hal-01204145

https://hal.archives-ouvertes.fr/hal-01204145

Submitted on 27 May 2020

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A Novel Viable Allele of Arabidopsis CULLIN1 Identified in a Screen for Superroot2 Suppressors by Next Generation Sequencing-Assisted Mapping

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Abstract

Map-based cloning (MBC) is the conventional approach for linking phenotypes to genotypes, and has been successfully used to identify causal mutations in diverse organisms. Next-generation sequencing (NGS) technologies offer unprecedented possibilities to sequence the entire genomes of organisms, thereby enabling direct identification of causal mutations without mapping. However, although mapping-by-sequencing has proven to be a cost effective alternative to classical MBC in particular situations, methods based solely on NGS still have limitations and need to be refined. Aiming to identify the causal mutations in suppressors of Arabidopsis thaliana superroot2 phenotype, generated by ethyl methane sulfonate (EMS) treatment, we combined NGS and classical mapping, to rapidly identify the point mutations and restrict the number of testable candidates by defining the chromosomal intervals containing the causal mutations. The NGS-assisted mapping approach we describe here facilitates unbiased identification of virtually any causal EMS-generated mutation by overlapping the identification (deep sequencing) and validation (mapping) steps. To exemplify the usefulness of the two approaches we discuss the strategy used to identify a new viable recessive allele of the Arabidopsis CULLIN1 gene in the non-reference Wassilewskija (Ws-4) accession.

Introduction

Map-based cloning (MBC) has been, and still is, widely used to identify genetic changes underlying mutant phenotypes in diverse organisms. It is a powerful technique with well-proven robustness [1], although traditional mapping experiments are generally labor intensive and hampered by needs for inter-accession crosses and selection of recombinants in the following generation(s) for mapping [2–4]. However, mapping mutations in well-established model organisms like Arabidopsis is much facilitated by the availability of a substantial genetic toolbox including an entire annotated reference genome, sequenced alternative accessions, and a multitude of marker systems [5]. In addition, several methods for identifying ethyl methane sulfonate (EMS)-induced point mutations in Arabidopsis and various other plant species have been developed using whole genome (re) sequencing following advances and reductions in cost of next generation sequencing (NGS) technologies. This has accelerated the process of identifying causal mutations, but methods based solely on NGS data still have limitations and need to be refined.

The NGS-based methods can be divided into two main types. The first is usually referred to as mapping-by-sequencing. The included methods all combine bulk segregant analysis (pooling recombinant genomes) with whole-genome sequencing (WGS) [1,6–10]. Their main advantage over classical MBC is that they allow simultaneous mapping and mutant identification, by analyzing NGS-generated data from a pool of recombinant F2 individuals and subtracting the putative causal mutations after comparing the sequences to a reference genome. Alternatively, bulked segregants in the same accession can be used for deep sequencing [11–14]. The second group of methods all use a more direct approach, direct sequencing of mutant genomes and subsequent identification of causative EMS-induced mutations by comparing them to a reference genome, thereby eliminating the need for outcrossing [15]. However, a reference genome is needed as a scaffold in both approaches, although a method based on comparing k-mers in WGS datasets that eliminates the need for segregating populations and reference sequences has been recently described [16].

The full potential of the abovementioned methods can only be exploited in particular situations (e.g. when two or more alleles are isolated in the same screen) and/or require specialized software. Here we used a reliable alternative that can be used when single or multiple alleles are identified in the same screen, in reference or...
The putatively causal mutation identified in the mapped region was subsequently confirmed using the reference genome sequence (TAIR10) using SOAP2 (http://soap.genomics.org.cn/soapaligner.html), and SNPs of the sequenced genome were detected using SOAPsnp (http://soap.genomics.org.cn/soapaligner.html). Using SNPs from the sur2-1gl1 sequence the corresponding sites in the TAIR10 were replaced, and the newly constructed sur2-1gl1 genome was subsequently used as reference for 494. To identify the 494-specific mutation, credible SNPs (which are likely mutations between 494 and sur2-1gl1) were filtered from credible loci differing between 494 and sur2-1gl1-TAIR10. These are relatively reliable loci filtered using the following criteria: consensus quality ≥20 (error rate ≤1%), total depth ≥5 and ≤50 (to avoid copy number variation, CNV), and estimate copy number of the site <2. Only homozygous 494 SNPs were considered further. We used the same approach to identify the causal mutation in the mutant 2035 (data not shown).

Confirmation of the Causal Point Mutation in 494 and Genotyping

The putatively causal mutation identified in the mapped region of 494 by analyzing the NGS dataset was subsequently confirmed using Sanger sequencing. To confirm the splicing defect and intron retention in the 494 mRNA, total RNA was extracted from seedlings of both 494 and sur2-1gl1 mutants using the RNAquous isolation kit (Ambion), treated with rDNase I, using

**Materials and Methods**

**Mutant Screen and Physical Mapping**

The superroot2 suppressor mutant designated 494 was identified in an EMS-mutagenized homozygous sur2-1gl1 population [17].
the DNA-free Kit (Ambion), and subsequently cDNA was synthesized using the iScript cDNA Synthesis Kit (BIO-RAD), according to the manufacturer’s instructions. Furthermore, gene-specific primers (Atg02570.F_TGGCTATCCGGCTCTTCTA and Atg02570.R_TTGCAACACACAACGACTT) spanning the splicing site altered by the 494 mutation were used to amplify the cDNAs, using standard PCR procedures.

To genotype the 494 point mutation, new derived cleaved-amplified polymorphic sequence (dCAPS) primers (494+EcoNL.E_GCCACTCTCTCCCTGCTTT) were designed using dCAPS Finder 2.0 software (http://helix.wustl.edu/dcaps/dcaps.html) [28]. One mismatch (underlined) was introduced in the F primer to incorporate a restriction site in the PCR product of one allele. After amplification, the PCR products were digested with EcoNI (Fermentas Fast Digest) following the manufacturer’s recommendations and electrophoretically separated on a 4% agarose gel.

Phenotypic Evaluation

The DNA-free Kit (Ambion), and subsequently cDNA was synthesized using the iScript cDNA Synthesis Kit (BIO-RAD), according to the manufacturer’s instructions. Furthermore, gene-specific primers (Atg02570.F_TGGCTATCCGGCTCTTCTA and Atg02570.R_TTGCAACACACAACGACTT) spanning the splicing site altered by the 494 mutation were used to amplify the cDNAs, using standard PCR procedures.

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To genotype the sur2-1, which carries a 61 bp insertion in the cytochrome P450 CYP38B1 gene [29], primers were designed (Sur2-1F_AGCTTGGTTTCGGACAGTACAC and Sur2-1R.AC TTAGATCAACCAGCTTGAT) that amplify a 237 bp fragment in wild type, and a 176 bp fragment in mutant, respectively.

To genotype the axr6-3 and cul1-7 alleles used for complementation tests, we used the genotyping primers and conditions described by Glinkerson et al. [24].

Phenotypic Evaluation

Mutants and corresponding wild-type plants cultivated in vitro were characterized and their auxin contents were quantified as previously described [17]. For phenotypic evaluation of soil-grown plants, seeds were first germinated in vitro then the resulting seedlings were transferred into pots, which were placed in growth chambers providing short day (8 h darkness/16 h light) conditions at 22 °C/18°C (light/dark temperatures). Plants under all growth conditions were visually inspected at time-points corresponding to selected developmental stages.

Results and Discussion

Mutant Isolation and Mapping

Genetic and physiological studies have shown that adventitious root (AR) formation is a heritable quantitative genetic trait controlled by multiple endogenous and environmental factors (reviewed in: [30–33]). Little is known about the molecular mechanisms controlling this developmental process, but we have recently started to unveil the complex regulatory mechanisms controlling AR formation. Using Arabidopsis thaliana as a model, we have shown that auxin and light signaling play essential roles in regulating AR formation on Arabidopsis hypocotyls [34–36]. We have also obtained preliminary indications that different regulatory pathways control lateral root and AR initiation in the hypocotyl, although both types of roots originate from pericycle cells [37,38].

To substantiate these findings, we screened seedlings obtained from EMS-mutagenized homozygous superroot2-1glabra1 (sur2-1gl1) seeds, aiming to identify Arabidopsis mutants that produce a nearly normal main root system but have specific impairment in AR formation on etiolated hypocotyls [17]. The mutant designated 494 was identified during that screen. The mutation it carries suppresses the AR phenotype of the superroot2-1 (sur2-1) mutant [39], and the suppressor mutant develops significantly fewer AR on the hypocotyl than sur2-1gl1, despite retaining similarly high endogenous IAA levels [17].

Homozygous 494 mutant plants selected from a homozygous M4 population were backcrossed four times with the sur2-1gl1 parental genotype to remove EMS-induced SNPs not associated with the phenotype. In parallel, homozygous 494 M3 mutant plants were outcrossed with the atr4-7 mutant, carrying an allele of the sur2 mutant in Col-0 background [26], to generate a mapping population. Lastly, to identify potential alleles, alleleic tests were conducted by crossing the mutant with other mutants isolated in the screen.

The 494 mutation was mapped on genomic DNA extracted from phenotyped mutant seedlings that produced fewer AR than sur2-1gl1, identified in a segregating F2 mapping population grown in vitro as previously described [5]. Coarse mapping was completed using newly identified INsersions/DEletions (INDEL) markers, as described in Materials and Methods. A flowchart of the NGS-assisted mapping approach, which can be applied for identifying mutations in both reference and non-reference Arabidopsis accession backgrounds, is shown in Figure 1.

Identification of the Putative Causal Mutation in the Mapped Chromosomal Region

Even after applying the filtering regime described in Materials and Methods we identified 33 mutations scattered across the five chromosomes by comparing the 494 and newly constructed sur2-1gl1 reference sequences, 25 of which were canonical C:G-to-T:A EMS-induced changes (Table S3 in File S1). Twenty-one of these mutations are situated in non-coding sequences (one in a 3’UTR, one in a 5’UTR, two in transposons, four in introns, and 13 in intergenic regions). Of the 12 mutations affecting the CDS, only six were non-synonymous. Interestingly, the only mutation located in the genomic interval 1.062.516 bp - 2.821.733 bp defined by the mapping markers UPSC_4_1062 and UPSC_4_2921 in the top of chromosome 4, is a synonymous G-to-A substitution at position 1.130.414 bp (Figure 2A). That mutation, located at the junction between the 5th exon and the 6th intron of the CUL1 gene (AT4G02570), affects the splicing efficiency of the 6th intron, as confirmed by comparing the PCR amplification products of the sur2-1gl1 and 494 cDNAs (Figure 2B). Gene-specific primers spanning the splicing site amplified a 463 bp fragment in sur2-1gl1, while in addition to the correct spliced variant a larger 551 bp amplicon was detected in 494 (Figure 2B). The size difference of 88 bp between the 494 amplicons corresponds to the size of the 6th intron. These observations show that although correct splicing occurs in 494 and some wild-type protein is produced, (probably less efficiently than in sur2-1gl1), the 494 point mutation leads to intron retention in the 494 mRNA, translation of which is predicted to yield a truncated protein product.

EMS mutagenesis typically induces hundreds of randomly distributed mutations per genome [3,15] that can hamper the direct identification of causal mutations when using NGS data alone. Performing sufficient alleleic tests (using existing alleles or T-DNA insertion lines), or complementing all of the candidates by transforming mutants with corresponding wild-type sequences would clearly be extremely tedious and generally unfeasible. The number of candidate mutations can be reduced by repeated backcrossing to the parental genotype before sequencing or by directly sequencing genomes carrying two or more independent mutations located in the same gene in multiple genomes. The first (repeated backcrossing) option is time-consuming, even if the focal organism has short generation times like Arabidopsis, and not always very efficient for removing EMS-induced SNPs that are not
associated with the phenotype of interest. However, backcrossing is not essential for identifying EMS-induced mutations through NGS-assisted mapping, nor is the number of backcrosses important, since the strategy yielded very similar results when applied to mutants backcrossed four or two times (Table S3 and Table S4 in File S1; [40]). In the example discussed here, a high number of potential candidate mutations were identified after filtering, even if mutant plants were backcrossed four times before sequencing. Second, a typical non-saturating EMS mutagenesis screen provides the likelihood of detecting only single alleles [41], therefore direct sequencing of the mutant will have to be supported by mapping [12,42]. Furthermore, in our particular

Figure 1. Identification of EMS-induced mutants by NGS-assisted mapping. For mutants identified in the reference Col-0 background, taking the green path leads to mutation identification. For mutants identified in a non-reference background, a parallel sequencing of the parental genotype (black path) is required. *Replacement of Col-0 specific SNPs with sites specific for the parental genotype, followed by use of the new constructed genome to extract the EMS-induced mutations in the mutant.

doi:10.1371/journal.pone.0100846.g001

Figure 2. Annotation of putative causal mutations (A). Locations of EMS-induced mutations affecting the CDSs on chromosome IV are marked with asterisks. The red asterisk indicate the mutation located in the region defined by mapping, which is flanked by the UPSC mapping markers [5]. Below, the structure of the CUL1 gene is shown, indicating positions of known recessive mutations giving viable mutants used in this study for complementation tests. The position and nature of the cul1-494 mutation, compared to wild type are highlighted on the DNA sequence. PCR amplification of the 494 and sur2-1gl1 cDNAs with primers spanning the splicing site affected by the cul1-494 mutation (B). A 463 bp fragment, corresponding to the correct spliced variant was detected in sur2-1gl1, while an additional 551 bp splicing variant was detected in 494.

doi:10.1371/journal.pone.0100846.g002
case we found that mapping-by-sequencing is less suitable for identifying mutations with a weak phenotypic penetrance because it is difficult or even sometime impossible to trace mutant seedlings correctly in pooled F2 recombinants, or contaminants that occasionally occur [5]. Thus, sequencing such pools could have generated misleading or truncated information as described by [40] and sequencing a pool of homozygous F2 mutants turned to be a more reliable alternative.

A New Viable Recessive Allele of the Arabidopsis CUL1 Gene Identified in a Screen for Superroot2 (Sur2) Suppressors

Confirmation by Sanger sequencing of the point mutation identified in the CUL1 gene (AT4G02570), the only mutation situated in the mapped region, prompted us to consider it as a strong candidate for the 494 suppressor phenotype and we named this new allele cul1-494.

To demonstrate unambiguously that the cul1-494 mutation confers the suppressor phenotype we conducted complementation tests using three viable Col-0 alleles axr6-3 [23], cul1-6 [18], and cul1-7 [24]. They all failed to complement cul1-494, confirming that cul1-494 is a new viable allele of CUL1. Moreover, both axr6-3sur2-1 and cul1-7sur2-1 double mutants produce fewer AR than sur2-1 (Figure 3), confirming that the axr6-3 and cul1-7 mutations suppress the AR phenotype of sur2-1 in a similar way to cul1-494.

Phenotypically, cul1-494 mutant differ in several respects from those carrying other known viable CUL1 mutant alleles, notably its developmental defects are less pronounced. The first allele, axr6-3 was isolated in a screen for ir1-1 enhancers designed to identify genes required for SCFTIR1-mediated auxin responses [23]. axr6-3 plants exhibit impaired auxin responses, reduced apical dominance, delayed senescence, reduced male fertility, and aberrant flower development. In addition, this allele is temperature-sensitive, mutant plants being sterile at 22°C, but fertile at 18°C. The axr6-3 point mutation, located at the N terminus of the protein, interferes with Aux/IAA protein degradation and prevents the assembly of SCFTIR1 complexes by disrupting ASK1 binding. cul1-6, a recessive viable allele that affects interaction with the SCF regulatory protein CAND1 (CULLIN ASSOCIATED AND NEDDYLYATION DISSOCIATED), was isolated in a screen for mutants resistant to sirtinol [10]. cul1-6 plants have defects in seedling and adult morphology, including delayed leaf emergence, retarded root growth, reduced apical dominance, curled leaves and altered floral morphology. In addition to reduced auxin sensitivity, cul1-6 seedlings display reduced sensitivity to other hormones including jasmonic acid, the cytokinin 6-benzyladenine and ethylene, and are hyposensitive to red and blue light [10]. The third, viable, missense, recessive Col-0 CUL1 allele, cul1-7, was identified from a screen designed to isolate mutants with defective degradation of an Aux/IAA-luciferase (IAA1-LUC) fusion protein [24]. The cul1-7 mutation affects submit interaction at the CUL1 C-terminus. The mutant displays pleiotropic developmental defects similar to axr6-3 and cul1-6 (dwarfed with reduced apical dominance and curly leaves). Another viable cul1 allele with a point mutation located at the C-terminus, icu13, recently isolated in the Enkheim-2 (En-2) background, shows developmental defects that have been associated with alterations of auxin signaling [25]. Traits of the mutant plants include mild leaf hyponasty, increased numbers of vegetative leaves, early bolting (with short flower stems), and reduced apical dominance relative to wild-type.

The suppressor mutant 494 was identified based on its AR-related phenotype, and characterized in connection to this developmental process [17]. A comparison of four-day old in vitro etiolated seedlings of 494 and cul1-494 mutants with axr6-3, cul1-7, cul1-6 and the parental controls Ws-4, sur2-1gl1 and Col-0, respectively, is shown in Figure 4A. Both 494 [17] and cul1-494 mutants displayed a reduced apical hook, like axr6-3 and cul1-6. However, seven days after transfer to light both 494 and cul1-494 grew better in vitro than axr6-3, cul1-7 and cul1-6 (Figure 4B).

When grown in soil cul1-494 flowers earlier than its control Ws-4 (Figure 4C), and like axr6-3 [23] has reduced male fertility. In contrast, 494 is fertile, and except for a reduced number of rosette leaves and slightly reduced height, does not display any other obvious developmental defects, despite retaining similar endogenous auxin contents to sur2-1gl1 [17]. These finding show that the cul1-494 mutation not only suppresses the AR formation but also other developmental defects associated with high auxin content, such as epinastic cotyledons and leaves, long petioles and small leaf blades (Figure 4B and C). The cul1-494 single mutant produces similar numbers of AR and lateral roots to the corresponding wild type, Ws-4, while axr6-3, cul1-7, cul1-6 do not develop AR and produce fewer lateral roots than the wild type (Figure 4B). These observations suggest that cul1-494 is a weaker allele than the others. Nevertheless, the suppression of AR production in 494 is likely due to weak auxin responses (since AtCUL1 is a component of SCF-type ubiquitin ligase complexes containing the F-box TIR1, which are essential for auxin responses [43]) arising from functional perturbation of SCF, as observed in the other mutants [18,23–25]. This hypothesis is consistent with our previous findings that expression of three auxin-inducible GH3 genes (GH3.3, GH3.5, and GH3.6) is strongly down-regulated in 494 suppressor mutant, despite its elevated endogenous auxin content [17]. These three GH3 genes are essential for AR development in Arabidopsis hypocotyls [35,36] and their expression level correlates with the number of ARs [17].

The cul1-494 mutant shares, to a certain extent, the pleiotropic perturbations associated with the other viable CUL1 mutant alleles and, more specifically, the fertility defect. AtCUL1 is a component not only of SCF-type ubiquitin ligase complexes containing the F-box TIR1 essential for auxin responses [43], but also complexes containing COI1, an F-box protein that mediates jasmonate signaling [44]. Furthermore, analyses of effects of point mutations in AtCUL1 have shown it plays an essential role in responses to jasmonates [18], [45], which are important regulators of plant development.

Figure 3. axr6-3 and cul1-7 mutations reduce the AR numbers produced by double mutants with sur2. The number of adventitious roots was counted on at least 35 seedlings of each line in two replicates and the data were pooled. Error bars indicate standard errors. One-way ANOVA and Tukey’s multiple-comparison post-tests indicate that the double mutants are not significantly different from their respective wild types (P<0.05; n>70).

doi:10.1371/journal.pone.0100846.g003
development and responses to environmental stresses [46]. Jasmonic acid (JA) has demonstrated importance for flower development and fertility [47], [48], and we recently showed that it inhibits AR formation downstream of auxin signaling in Arabidopsis hypocotyls [36]. Therefore, the low number of ARs produced by the suppressor mutant is most likely due to a defect in SCFTIR1 functions, but not in SCFCOI1 functions.

Our recent publication indicates that, in addition to auxin responses, ethylene biosynthesis is potentially impaired in the mutant [17]. Interestingly, cul1-6 also reportedly has reduced sensitivity to ethylene [18], and disturbed responses to several other hormones known to require SCF function have been observed in different CUL1 mutants [18], [24]. This is consistent with observations that all the hormones known to be involved in the control of AR formation interact through complex crosstalk [33], which remains to be fully elucidated.

Conclusions

To our knowledge, only two reports describe the identification of either a spontaneous [49] or EMS induced point mutation [15] in non-reference Arabidopsis accessions by NGS, and none promote the advantages of combining MBC with NGS to identify point mutations. Liu et al. [50] have used targeted parallel sequencing of defined genomic regions to identify Arabidopsis mutants, but although their method combines MBC with deep sequencing we found more advantageous to combine NGS and mapping. Our strategy has potentially the broadest applicability in Arabidopsis analyses for several reasons. Firstly, coarse mapping to the Arabidopsis genome is now straightforward, and facilitated by recent updates to TAIR (The Arabidopsis Information Resource; http://www.arabidopsis.org/) marker database [5]. Secondly, NGS and bioinformatics (which do not require extensive prior training to interpret), can be acquired at competitive costs by

Figure 4. Seedling and rosette phenotypes of the and cul1-494 mutants. Seedlings were first etiolated in the dark for four days (A) and then transferred to the light for seven days (B). For phenotypic evaluation of soil-grown plants, seeds were first germinated in vitro and the resulting seedlings were subsequently transferred into pots, which were placed in growth chambers providing short day (8 h darkness/16 h light) conditions, at 22°C/18°C (light/dark temperatures) (C). Arrowheads indicate the root–hypocotyl junction; arrows indicate adventitious roots. Bars, 1 cm. doi:10.1371/journal.pone.0100846.g004
commercial service providers. Thirdly, combining the output of the two parallel complementary strategies can straightforwardly identify causal mutations.

The cited literature shows the unprecedented advantages of using NGS technologies to identify point mutations in various genetic backgrounds and model systems [1–7,16]. The multitude of methods developed by various research groups [1,7–16] shows the versatility of NGS data sets, and the advantage of using them in combination with either established or new developed algorithms for mutation identification. Several recent articles describe the use of NGS data, exclusively, to identify causal mutations in EMS-induced mutants and eliminate reliance on labor-intensive classical mapping [1,12]. However, although they provide examples of success, they also acknowledge the limitations of using NGS alone. Here we have shown that even in the NGS era classical mapping still provides valuable, complementary and robust data. The published strategies that completely exclude mapping may not be suitable in particular situations like the one we faced with low penetrant phenotype and only one allele identified. Arabidopsis mapping experiments are easily set up, and turned to be a valid option for defining genetic intervals containing causal mutations. The NGS-assisted mapping approach we describe here combines known techniques and provides a highly reliable alternative when only one allele is available [42].

The *cul1-494* allele we identified using NGS-assisted mapping is a valuable addition to the collection of weak-allele *CUL1* mutants, which will likely contribute to dissections of the role of *CUL1* protein in plant development. It may be particularly helpful for studying the hormonal and ethylene signaling cross talk involved in AR formation in Arabidopsis.

**Supporting Information**

**File S1** Contains Table S1, Summary of the sequencing data production (Clean_Data). Table S2, Summary of the alignment results. Table S3, Annotation of homozygous mutations identified by sequencing the genome of 494 after four backcrosses. In bold, the causal suppressor mutation. Table S4, Annotation of homozygous mutations identified by sequencing the genome of 2035 after two backcrosses. (DOCX)

**Acknowledgments**

The authors would like to acknowledge the support of Nicolas Delhomme and Chizanaka Madzurumwa Manapperuma ([http://bioinformatics.ruc.se](http://bioinformatics.ruc.se)) for handling the NGS dataset. We are very grateful to Marcel Quint (Leibniz Institute of Plant Biochemistry), Mark Estelle (UC San Diego) and Judy Callis (UC Davis) for kindly providing the *avr6-3*, *ahl-6* and *ahl-7* mutant lines.

**Author Contributions**

Conceived and designed the experiments: DIP MLP CB. Performed the experiments: DIP MLP AMP LG. Analyzed the data: DIP. Wrote the paper: DIP.

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