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## Methylation status of Arabidopsis DNA repair gene promoters during Agrobacterium infection reveals epigenetic changes in three generations

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**Abstract:**

Agrobacterium tumefaciens is a unique pathogen with the ability to transfer a portion of its DNA, the T-DNA, to other organisms. The role of DNA repair genes in Agrobacterium transformation remains controversial. In order to understand if the host DNA repair response and dynamics was specific to bacterial factors such as Vir proteins, T-DNA, and oncogenes, we profiled the expression and promoter methylation of various DNA repair genes. These genes belonged to nucleotide excision repair (NER), base excision repair (BER), mismatch repair (MMR), homologous recombination (HR) and non-homologous end joining (NHEJ) pathways. We infected Arabidopsis plants with different Agrobacterium strains that lacked one or more of the above components so that the influence of the respective factors could be analyzed. Our results revealed that the expression and promoter methylation of most DNA repair genes was affected by Agrobacterium and, it was specific to Vir proteins, T-DNA, oncogenes or just mere presence of bacteria. In order to determine if Agrobacterium induced any transgenerational epigenetic effect on the DNA repair gene promoters, we studied the promoter methylation in two subsequent generations of the infected plants. Promoters of at least three genes, CEN2, RAD51, and LIG4 exhibited transgenerational memory in response to different bacterial factors. We believe that this is the first report of Agrobacterium -induced transgenerational epigenetic memory of DNA repair genes in plants. In addition, we show that Agrobacterium induces a short-lived DNA strand breaks in Arabidopsis cells, irrespective of the presence or absence of virulence genes and T-DNA.


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# Methylation status of *Arabidopsis* DNA repair gene promoters during *Agrobacterium* infection reveals epigenetic changes in three generations

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## KEY MESSAGE:

- *Agrobacterium tumefaciens*, a widely used genetic engineering tool, altered the expression and promoter methylation of DNA repair genes, and induced short-lived DNA strand-breaks in *Arabidopsis*.
- The expression and epigenetic changes were specific to various *Agrobacterium*-derived factors.
- The promoter of three genes exhibited transgenerational memory in response to *Agrobacterium*-derived factors.

## ABSTRACT

*Agrobacterium tumefaciens* is a unique pathogen with the ability to transfer a portion of its DNA, the T-DNA, to other organisms. The role of DNA repair genes in *Agrobacterium* transformation remains controversial. In order to understand if the host DNA repair response and dynamics was specific to bacterial factors such as Vir proteins, T-DNA, and oncogenes, we profiled the expression and promoter methylation of various DNA repair genes. These genes belonged to nucleotide excision repair (NER), base excision repair (BER), mismatch repair (MMR), homologous recombination (HR) and non-homologous end joining (NHEJ) pathways. We infected *Arabidopsis* plants with different *Agrobacterium* strains that lacked one or more of the above components so that the influence of the respective factors could be analyzed. Our results revealed that the expression and promoter methylation of most DNA repair genes was affected by *Agrobacterium* and, it was specific to Vir proteins, T-DNA, oncogenes or just mere presence of bacteria. In order to determine if *Agrobacterium* induced any transgenerational epigenetic effect on the DNA repair gene promoters, we studied the promoter methylation in two subsequent generations of the infected plants. Promoters of at

least three genes, *CEN2, RAD51,* and *LIG4* exhibited transgenerational memory in response to different bacterial factors. We believe that this is the first report of *Agrobacterium*-induced transgenerational epigenetic memory of DNA repair genes in plants. In addition, we show that *Agrobacterium* induces a short-lived DNA strand breaks in *Arabidopsis* cells, irrespective of the presence or absence of virulence genes and T-DNA.

**Key words:** DNA repair, epigenetic memory, methylation, DNA strand break, *Agrobacterium*, *Arabidopsis*

## INTRODUCTION

*Agrobacterium tumefaciens* is a soil borne plant pathogen that causes crown gall disease. Apart from being a pathogen, its use in genetic engineering has garnered attention from scientists. This bacterium is termed natural genetic engineer because of its ability to transfer a stretch of its own plasmid DNA, known as the T-DNA, into the plant host cells. This ability of *Agrobacterium* for inter-kingdom gene transfer has been highly appreciated and exploited for the development of transgenic plants. Recently, *Agrobacterium* is also used to transfer genome editing reagents into plants (Sardesai and Subramanyam 2018). Interestingly, hosts compliment the process of T-DNA integration by providing an array of DNA repair proteins. In order to understand whether the T-DNA integration deploys the non- homologous recombination (NHR), homologous recombination (HR), or any other pathway, many studies were performed in plant and yeast model systems. For example, genes such as *KU80, MRE11, LIG4, XRS2, SIR4,* and *RAD50* were reported to facilitate *Agrobacterium*-mediated transformation *via* various NHR pathways (van Attikum et al. 2001; van Attikum and Hooykaas, 2003; Friesner and Britt, 2003; Li et al. 2005). Mestiri et al. (2014) reported that the absence of genes belonging to NHR pathways (*KU80, XRCC1* and *XPF*) as well as the HR pathway (*XRCC2*), individually or in combinations, significantly reduced T-DNA integrations. Though the simultaneous absence of all the four genes minimised the number of T-DNA integrations, it could not completely prevent the integration, suggesting that there is/are alternate pathway(s) involved in T-DNA integration. In contrast to the above results, reports by Vaghchhipawala et al. (2012) and Park et al. (2015) indicated that four genes (*KU70*, *KU80*, *DNA LIG6* and *XRCC4*) belonging to the non-homologous end joining (NHEJ) pathway, increased stable transformation and T-DNA integration. Subsequently, there were reports that polymerase theta (Pol θ) could be a key player in T-DNA integration (van

Kregten et al. 2016). Pol θ-dependent T-DNA integration requires microhomology, and the fact that T-DNA integration is often accompanied by deletions, insertions, filler sequences, translocations, recombinations and vector back-bone integrations (Gelvin 2017; Majhi et al. 2014), it was proposed that there are other parallel DNA repair pathways involved in T-DNA integration (Gelvin 2017; Lacroix and Citovsky 2019). In order to identify what other parallel DNA repair pathways are involved in T-DNA integration, it is important to initiate an elaborate profiling of the host DNA repair system under the influence of *Agrobacterium*.

*Agrobacterium* is known for its unique factors Vir proteins, T-DNA and oncogenes, which it transfers into plant cells. Previous reports show that the host plants exhibit differential expression of defense-related genes in response to *Agrobacterium* pathogen associated molecular patterns (PAMPs), Vir proteins and T-DNA. For example, Vir proteins suppressed the expression of many defense related genes in the host (Veena et al. 2003; Ditt et al. 2005). Similarly, differential response to *Agrobacterium* Vir proteins was observed in terms of host genome stability wherein the presence of Vir proteins led to the suppression of various mutations such as transversions, somatic homologous recombinations (SHRs), and frame-shift mutations (Shah et al. 2015). It could be possible that the expression of host DNA repair genes was also specific in response to the unique factors of *Agrobacterium*.

There are several reports of host gene expression profiling after *Agrobacterium* infection but these reports mostly used oncogenic *Agrobacterium* strains and reported more on host defense and other responses (Deeken et al. 2006; Ditt et al. 2006; Lee et al. 2009). Oncogenes induce tumors and hence greatly influence host gene expression. However, they are not required for T-DNA integrations. Hence, studies involving oncogenic *Agrobacterium* strains could mask the events specific to T-DNA integration. Macroarray analysis using virulent and avirulent *Agrobacterium* strains by Veena et al. (2003) was done on tobacco cell cultures and this system does not represent an individual plant. Also, this report revealed the expression of defense genes, various histone-encoding genes and others, but not DNA repair genes. It is also known that the host response to pathogens is very dynamic and keeps changing with the period of infection (Kuśnierczyk et al. 2008; Veena et al. 2003; Wang et al. 2005). Studies focusing on the dynamics of host DNA repair gene expression under the influence of specific *Agrobacterium*-derived factors (T-DNA, Vir proteins and oncogenes) are lacking.

Most of the previous whole genome expression profiling experiments in plants under *Agrobacterium* infection focused on gene expression in the infected cells (Veena et al. 2003; Deeken et al. 2006; Ditt et al. 2006 and Lee et al. 2009). It is known that plants exhibit a

systemic response to pathogens and it is not just the infected cells, but distal cells also respond to pathogens (Deleris et al. 2016; Heil and Ton 2008). Considering this and the fact that *Agrobacterium* is motile (Tomlinson and Fuqua 2009), the gene expression in distal cells also has to be studied so that the general response of host could be understood.

Epigenetic changes are crucial regulators of gene expression and DNA methylation is a major epigenetic marking system in plants (Zhang et al. 2018). Microbes such as virulent and avirulent *Pseudomonas* strains and *Tobacco mosaic virus* (TMV) trigger extensive DNA methylation and demethylation in the host genome and much of these studies focussed on host defence and immune response (Deleris et al. 2016). Previously, increased global genome methylation and decreased methylation of promoters in general, was observed in *Agrobacterium*-induced crown galls of *Arabidopsis* (Gohlke et al. 2013). Other epigenetic studies involving plant-*Agrobacterium* interaction confined to the integrated T-DNA or transgene (Gelvin et al. 1983; Linne et al. 1990; Kilby et al. 1992; Philips et al. 2019; Jupe et al. 2019). It could be possible that some of the host DNA repair genes are epigenetically regulated under the influence of *Agrobacterium* or its unique factors and this is to be elucidated. Epigenetic changes can be transferred to the progeny (intergenerational) and even grand-progeny (transgenerational) (Deleris et al. 2016; Heard and Martienssen 2014; Lämke and Bäurle 2017). Previously Boyko et al. (2007) reported that TMV infection hypomethylated many LRR-containing loci in *Nicotiana tabacum* plants and some of these changes was retained in the progeny as well. There are no reports on such epigenetic memory induced in plants due to *Agrobacterium*.

In this report, we studied the expression and promoter methylation of DNA repair genes belonging to various pathways such as nucleotide excision repair (NER), base excision repair (BER), mismatch repair (MMR), HR, and NHEJ in *Arabidopsis* plants infected with four different *Agrobacterium* strains. These *Agrobacterium*-derivatives differed for the presence of various factors (Vir proteins, T-DNA, oncogenes, and PAMPs). We considered the influence of these unique factors and various infection time intervals simultaneously, so that the dynamics of expression and methylation patterns could be studied elaborately. Apart from the previously reported NHEJ and HR genes, expression of genes from all other DNA repair pathways was also altered in our study. Altered promoter methylation was also seen in many of these genes from all the pathways. Interestingly, the change in expression as well as promoter methylation of each gene was specific to any of the above-mentioned unique bacterial factors. We extended the promoter methylation analysis to progeny generations and this revealed that some of the DNA repair genes exhibited transgenerational epigenetic

memory. The expression and epigenetic profile thus created gives an elaborate picture of the host DNA repair system under the influence of *Agrobacterium*. Since DNA repair is associated with genome stability, we studied the host DNA strand breaks in plants under the influence of various bacterial factors.

## MATERIALS AND METHODS

### *Agrobacterium* culture conditions and strains used

The *A. tumefaciens* strains used in this study include the wild-type Ach5 (referred to as VOT in the main body of the paper) and its derivatives (Table 1) grown on Luria-Bertani (LB) media containing appropriate antibiotics (Table 1). A loop of bacterial culture was inoculated into liquid LB broth and kept at 24 °C with shaking at 220 rpm for overnight. Liquid cultures with optical density 0.8-1 at 600 nm were used for infection.

### *Arabidopsis* growth conditions and infection method

*Arabidopsis thaliana* (ecotype Col-0) seeds were surface sterilized and plated equidistantly on MS (Murashige and Skoog) medium containing 3% sucrose, pH 5.7. Uniform germination of seeds was achieved by placing the plates in the dark at 4°C for 48 h. For infection, bacterial culture was inoculated at the base of the petiole adjoining the node of four-week-old *Arabidopsis* plants by making wounds (4-5 pricks) using 2 ml sterile clinical syringe. Plants that received blank LB medium without any *Agrobacterium* served as mock-infected controls. For expression analysis, infections were performed on four-week-old plants. In experiments involving plant epigenetic memory or comet assay, three-week-old plants were infected and subjected to DNA extraction or nuclei isolation, respectively. For the purpose of epigenetic analysis, which involves transgenerational memory, a parallel set of infected and control plants were carefully transferred from MS media to vermicompost obtained from CPCRI (Central Plantation Crops Research Institute, Kasaragod, India), one week after infection. Seeds collected from these plants ($2^{nd}$ generation) were again grown on MS medium for three weeks and subjected to DNA extraction. Similar to the first generation, four-week-old plants were carefully transferred to vermicompost to obtain the third generation plants. The above steps were repeated for the third generation as well. Plants on MS media or vermicompost were raised in a growth chamber (Percival, USA. Model Ar-3663), under uniform conditions of light (8000 lux), temperature (24°C) and humidity (100%) with a 16 h light/8 h dark cycle.

### Beta-glucuronidase (GUS) histochemical staining

Infected and mock-infected plants were incubated at 37°C for 48 h in GUS-staining solution [100 mM sodium phosphate buffer (pH 7.0)] containing 1 mM 5-bromo-4-chloro-3-indolyl glucuronide (X-Gluc) and 0.1% Triton X-100 (Jefferson 1989). Subsequently, these plants were bleached with 70% ethanol. The stained portions were imaged using a Stemi DV4 stereomicroscope.

**Selection of *Arabidopsis* DNA repair genes, promoter region identification, primer design and methylation-sensitive restriction enzyme (MSRE) site identification**

Nucleotide sequences of 22 DNA repair genes and their promoters were accessed from NCBI (https://www.ncbi.nlm.nih.gov). Plant Promoter Data Base-PPDB version 3.0 (http://www.ppdb.gene.nagoya-u.ac.jp) was used for finding the promoter regions of DNA repair genes in *Arabidopsis*. Primers (Table S1) were designed using the online tool on the Integrated DNA Technology (IDT) website, https://www.idtdna.com. For expression analyses, primers were designed from the coding region, flanking introns, as described in Joseph et al. (2018). For methylation-sensitive restriction digestion (MSRD)-PCR, primers were designed from the promoter region flanking four or more methylation-sensitive restriction enzyme (MSRE) sites (Fig. 1; Fig. S1). MSRE sites in the promoter sequences were identified using NEB cutter (https://www.neb.com/tools-and-resources/interactive-tools/double-digestfinder).

**Extraction of DNA and RNA, their quantification, and cDNA synthesis**

Total DNA or RNA was extracted from the aerial parts (leaves and petiole 2-5 mm away from the site of infection) of about 17 or 10 plants respectively, under each treatment. Total genomic DNA was extracted using the CTAB (Cetyltrimethylammonium Bromide) method (Rogers, 1988). RNA was extracted using Trizol reagent (Invitrogen, USA) and subjected to DNase I (Genie, India). Nanodrop 2000c Spectrophotometer (Thermo Scientific, India) was used to evaluate the quantity and quality of extracted DNA and RNA samples. An equal amount of total RNA (1 µg) was taken for all the treatments and its cDNA was synthesized using a *Moloney murine leukemia virus* Reverse Transcriptase (M-MLV-RT) kit (Invitrogen, USA) according to manufacturer's instruction. Synthesized cDNA was made free of RNA by treating with RNase H (Invitrogen, USA).

**Standardisation of PCR conditions and verification of amplicon sizes and sequences**

Gradient PCR on Eppendorf MasterCycler was carried out to determine the best working temperature for the primers. The PCR cycles consisted of an initial denaturation at 95°C for

10 min, followed by 40-50 PCR cycles at 95°C for 40 sec, different primer annealing Tm (Table S1) for 40 sec and 72°C for 40 sec and, a final extension at 72°C for 5 min. The amplicon sizes with primers to be used for real-time quantitative RT-PCR (qRT-PCR) or MSRD-PCR, were verified on 2% agarose or 0.8-1.2% agarose gels, respectively. Fragments of the expected sizes from cDNA (Fig. S2) and DNA (Fig. S1) for expression and methylation analyses, respectively, were obtained. As the primers used for qRT-PCR flanked an intron, the larger band observed in genomic DNA and smaller band in cDNA (Fig. S2 a to e, g, I to j and v) confirmed that the RNA extracted did not have any DNA contamination. For nucleotide sequence verification, PCR products were sequenced at Xcelris labs Limited India and AgriGenome labs Private Limited, India.

**Real-time quantitative RT-PCR (qRT-PCR) and analysis of gene expression**

PCR reactions were conducted on a Roche-LightCycler® 480 II system using Essential DNA Green Master (Roche Diagnostics, USA). The concentration of cDNA template was kept uniform by normalizing the concentration of RNA. Final primer concentration was 1 picomole/reaction. The thermal cycle consisted of an initial denaturation at 95°C for 10 min, followed by 45 PCR cycles at 95°C for 30 sec, different primer annealing Tm (Table S1) for 15 sec and 72°C for 15 sec. Each PCR cycle was followed by fluorescence acquisition at 95°C for 1 min and 65°C for 1 min. Subsequently, a melting curve was generated by increasing temperature from 65 to 95°C, in order to verify primer specificity. Cp (crossing point) values were generated by the LightCycler® 480 SW 1.5.1 software with default parameters. Three independent experiments (biological replicates) were conducted for each gene and, triplicates of each treatment condition (technical replicates) were conducted in each experiment. We had previously identified *UBQ10* as the suitable reference gene for normalization under all the treatment conditions under study (Joseph et al. 2018) and hence this gene was used for expression analysis. The fold changes in the expression of candidate genes were assessed by $2^{-\Delta\Delta Ct}$ value obtained by qRT-PCR. The heat map of expression profile of genes was generated using QCanvas 1.21 software (compbio.sookmyung.ac.kr/~qcanvas/index.html).

**Statistical analysis**

The fold change values of each treatment were subjected to a one-way Analysis of Variance (ANOVA, $\alpha = 0.05$) to determine significant changes ($P < 0.05$). Genes with significant

variations ($P$ < 0.05) were taken for post- hoc analysis using Tukey-HSD (Tukey, 1953) in Rstudio.

## Methylation-sensitive restriction digestion (MSRD)-PCR

The MSRD-PCR involved two steps: one, digestion using MSREs and two, PCR (Fig. 1). Complete digestion of DNA leads to absence of band in PCR, indicating absence of methylation and in contrast, lack of digestion/incomplete digestion leads to the presence of a band indicating methylation (Fig. 1). The optimum conditions for MSRE digestion was standardised by digesting genomic DNA with different concentrations (10 U/µg and 20 U/µg) of six MSREs (*Hpy*ChIV4, *Hpy*99I, *Bsa*AI, *Aci*I, *Cla*I, and *Ape*KI) under different incubation period (1 h and 2 h) at 37°C. All MSREs were obtained from New England Biolabs, USA. PCR was done using 50 ng of digested DNA and undigested DNA (as positive control). Appropriate conditions of enzyme concertation and digestion period for complete digestion were obtained by comparing results of multiple loci. For instance, *Aci*I site was present in *KU80* and *ATM* gene promoters but absent in *RAD23D* gene promoter (Fig. S1). Hence, PCR of DNA digested with *Aci*I was expected to give amplification in *RAD23D* but not in *ATM* and *KU80* gene promoters. Though no amplification was obtained from DNA digested for 1 h using 10 or 20 U/µg of MSRE with *KU80* promoter-specific primers, a faint amplicon was obtained using *ATM* promoter-specific primers from DNA digested with 10 U/µg of the enzyme (Fig. S3a). However, digestion using 10 U/µg of the enzyme for 2 h did not yield any amplification and hence, this enzyme concentration and digestion time was chosen for further experiments. Additionally, *RAD23D*-specific primers gave an amplification with the same digested DNA due to the absence of this site within the amplicon (Fig. S3a). This ensured that the quality of the DNA was good and the lack of amplification in *KU80* and *ATM* gene promoters was not due to DNA degradation. Similarly, the digestion and PCR conditions for all the remaining enzymes were optimized (Fig. S3). MSRD-PCR analysis was done in three generations of the infected plants. PCR of first generation was performed using DNA that was extracted from 17 plants together. Three sets of progenies obtained from three different plants were analysed in 2$^{nd}$ (G2-I, G2-II, G2-III) and 3$^{rd}$ (G3-I, G3-II, G3-III) generations in order to confirm the consistency in the results.

## Comet assay for DNA strand break detection

The nuclei were extracted from the infected zone (2-5 mm long segment) of about ten plants. The plant tissue was chopped into small pieces with a razor blade in 500 µl chilled phosphate

buffered saline (160 mM NaCl, 8 mM Na2HPO4, 4 mM NaH2PO4, pH 7.0) containing 50 mM EDTA, on ice (Menke et al. 2001; Vaghchhipawala et al. 2012). Nuclei suspension was collected into microfuge tubes on ice after removing tissue debris by filtration through 60 µm nylon mesh. Alkaline comet assay was performed to detect DNA strand breaks (Dikilitas 2009). Slides were prepared by mixing 500 µl of nuclei suspension in 1 ml of 0.8% low melting point agarose and uniformly layering this mixture on top of the slides that were pre-coated with 1% normal melting point agarose. After agarose solidification the slides were immersed overnight in freshly prepared, chilled lysing solution (1.2 M $NaCl_2$, 100 mM $Na_2EDTA$, 0.26 M NaOH, 0.1% SDS, pH > 13) at 4 °C. After lysis, the slides were washed for 20 min, thrice, in chilled alkaline electrophoresis buffer (0.03 M NaOH, 2 mM $Na_2EDTA$, pH ~ 12.3). Subsequently, electrophoresis was done in chilled alkaline electrophoresis buffer for 20 min at 0.7 V/cm (50 mA/25 V) at 4 °C. The slides were then gently washed with 0.4 M Tris buffer (pH 7.5) for neutralization. Afterward, the slides were immersed in propidium iodide stain (0.02 mg/ml) for 20 min in the dark. After staining, slides were washed in chilled distilled water to remove excess stain. Individual cells were examined under a fluorescence microscope, Leica DMI3000 B (excitation filter 515–560 nm and a barrier filter of 590 nm) at 20 X–40 X magnification.

## RESULTS

### Host DNA repair genes in distal tissues exhibit a dynamic expression change in response to various *Agrobacterium* factors

Prior to beginning our experiments on host DNA repair genes, we wanted to ensure that the method used for infection was efficient to transfer T-DNA. For this, we infected about 10 plants using disarmed *Agrobacterium* strain LBA4404 (pCAMBIA2301) that had a *GUS* gene under the constitutive CaMV-35S promoter within its T-DNA. We observed blue staining at the infection site (Fig. S4) in all the plants, 48 h after infection, and this confirmed that our method of infection was effective. In order to study if the DNA repair genes were influenced by various *Agrobacterium*-derived factors, the expression levels of 22 DNA repair genes in *Arabidopsis* plants infected with one of four different *Agrobacterium* strains designated as VOT, XXX, VXX, and VXT (Table 1), during three different post-infection intervals (4 h, 24 h, and 48 h), were assessed by qRT-PCR. All the four *Agrobacterium* strains belonged to Ach5 background and they differed for the presence or absence of the three unique factors; Vir proteins, oncogenes and T-DNA (Table 1). Hence, by comparing the response of host to

each strain, we would be able to deduce whether each of these factors had any influence. After infecting *Arabidopsis* plants at the base of petiole, aerial tissue (leaves and petiole) was collected, excluding the inoculation site, for RNA isolation and qRT-PCR. Fold change in gene expression for all the 22 genes under the proposed 12 different treatment conditions was calculated by $2^{-\Delta\Delta Ct}$ method (Table S2). Eighteen of 22 genes showed statistically significant differential gene expression during different time points. This proved that *Agrobacterium* infection modulates gene expression in distal tissue of *Arabidopsis* and that, the response of each gene was specific to certain *Agrobacterium* strains (Fig. 2; Fig. S5). For example, increased and decreased expression was seen for four (*LIG4, MRE11A, RAD4,* and *XRCC1*) and three (*BLM, MSH6,* and *MBD4*) DNA repair genes, respectively, in response to *Agrobacterium* strains with *Vir* genes (Fig. S5a, b, respectively), irrespective of the presence or absence of T-DNA and oncogenes. Hence, we grouped them into the category of genes affected by Vir proteins. Similarly, we could group genes in response to T-DNA, oncogenes and mere presence of bacteria (probably PAMP) (Fig. S5). These results thus confirmed that each of the unique bacterial factor analysed had some role in influencing the expression of host DNA repair genes.

Significant temporal changes in gene expression was also observed in these genes. Categorization of the affected genes based on their temporal response (Fig. S6) revealed that all the 18 genes invariably had altered expression 24 h post-infection apart from the changes that occurred in other time intervals. It was noted that the expression of most of the genes reverted to normal at 48 h post-infection (Fig. S5), except *XPF*. *XPF* was the only gene to be affected by oncogenes and was differentially expressed at 24 h and 48 h (Fig. S5d; Fig. S6). There was no gene whose expression was confined only to either 4 h or 48 h post-infection (Fig. S6). This suggests that plant-*Agrobacterium* interaction greatly influences the host DNA repair machinery between 4 h and 48 h of infection. We further categorized the gene responses based on the DNA repair pathway that they belong to (Fig. S7). While all infection conditions triggered a response in at least one NER gene, the response of genes from other pathways was confined only to selected infection conditions (Fig. S7). This indicates that there is probably a defined and non-overlapping mechanism for the regulation of various host DNA repair genes by *Agrobacterium* factors.

**Host DNA repair gene promoters exhibit temporal methylation/demethylation in response to different *Agrobacterium* factors**



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Promoter methylation status of the above-mentioned 22 DNA repair genes in tissue of plants distal from sites infected with various *Agrobacterium* strains was assessed by MSRD-PCR. Primers for MSRD-PCR (Table S1) were designed from the promoter region consisting of a minimum of four MSRE sites (Fig. 1, Fig. S1), 50-1400 bp before the start codon. The duration of methylation can vary from a few hours to many generations (Iwasaki and Paszkowski 2014). Hence, similar to our expression studies, infections were done for the three time intervals, 4 h, 24 h and 48 h. DNA was extracted from 17 plants under each treatment.

The presence/absence of methylation was determined based on the PCR amplification of DNA digested with appropriate MSRE. Presence of the band indicated methylation and its absence indicated the absence of methylation (Fig. 1). Sufficiently high amount of DNA from the same extraction was digested with different enzymes and PCR for all loci were conducted with the same digested DNA sample. Since the same DNA sample was methylated for some loci and demethylated or unaffected for others (Table S3), these DNA samples served as controls for methylated and unmethylated DNA.

Promoters of 14 of 22 genes had altered methylation status in any one or more enzyme sites (Table S3). Methylation and demethylation was seen in nine (*XAB2, RAD4, CEN2, BRCA2, PMS2L3, RAD51, LIG4, BLM,* and *TAG1*) and four (*RAD23B, KU70, RAD23D,* and *MSH6*) gene promoters, respectively. Promoter of one gene (*RAD23C*) was an example of both methylation as well as demethylation because it was demethylated by wounding and methylation was restored by all the *Agrobacterium* strains at 48 h post-infection (Table S3). The influence of different *Agrobacterium*-derived factors on methylation status was determined based on the strain used for infection (Fig. 3). Hence, *RAD23C* promoter was placed in two categories, one, methylated due to mere presence of bacteria and two, demethylated due to wounding (Fig. 3). In other examples, methylation and demethylation in *BRCA2* and *RAD23B* gene promoters, respectively (Fig. 3) was induced by strains with *Vir* genes. Hence, we inferred that Vir proteins could be the reason for the changes. Similarly, all the promoters were analyzed and the profile in response to various *Agrobacterium*-derived factors and infection time, in terms of methylation status was created (Table 2). From this, we could also categorise the genes based on the temporal influence of various bacterial factors (Fig. S9). This conveyed that none of the bacterial factors induced changes confining to 4h. Only Vir proteins induced an early temporary alteration as shown by the two NER genes (*RAD4* and *XAB2*). All bacterial factors as well as mock-infection induced changes that were retained at least up to 48 h post-infection (Fig. S9). These results also suggest that *Agrobacterium* infection alters methylation of gene promoters in distal tissues.

**Comparison of the promoter methylation status and expression of DNA repair genes**

DNA methylation/demethylation of promoters has a role in the regulation of gene expression (Zhang et al. 2018). Methylcytosines in the promoter regions are known to mostly repress transcription (Woo et al. 2007). However, in our experiments only two genes *KU70* and *RAD23C* followed this rule (Table 3). Expression of *KU70* was up-regulated and its promoter was demethylated due to the influence of T-DNA. Mere presence of bacteria (PAMP) lead to promoter methylation and reduced *RAD23C* expression. There were two exceptions in contrast to the above concept where, Vir proteins and T-DNA induced promoter methylation and enhanced the expression of *RAD4* and *RAD51*, respectively. In about eight genes (*MBD4, CEN2*, *LIG4, P3MAG1, XRCC1, MRE11A, XPF,* and *KU80*), the factor that lead to expression change did not lead to change in methylation status and vice versa (Table 3). Two other genes *TAG1* and *PMS2L3*, though had their promoters methylated in response to various infection conditions, they did not show any change in their expression under all the infection conditions.

***Agrobacterium* induces epigenetic memory in host DNA repair gene promoters**

In order examine if *Agrobacterium* or its factors could induce epigenetic memory in the promoter regions of the DNA repair genes, we wanted to repeat the methylation analyses in uninfected progeny generations. Hence, we independently subjected the petiole bases of three *Arabidopsis* plants (G1-I, G1-II, and G1-III) (Fig. 4) to four bacterial strains (VOT, XXX, VXX, VXT; Table 1) and mock infection (wounding). Seeds were collected from the above three G1 plants under each of the above treatment conditions as well as untreated controls. Three sets of seeds were obtained (G2-I, G2-II, and G2-III) from the three G1 plants (Fig. 4), under each treatment. These G2 seeds were germinated and about 17 plants from each G2 set (Fig. 4) were subjected to DNA extraction and MSRD-PCR. Methylation analysis of the same 14 DNA repair gene promoters, which had altered methylation status in the parental G1 generation, was repeated in the three sets under all treatment conditions. Interestingly, eight out of 14 promoters preserved their methylation/demethylation pattern in the progeny generation (Table 4; Fig. S8). Of the eight, three and five were examples of demethylation and methylation, respectively (Table 4). It is to be noted that for each promoter under one particular treatment, the number of plants analysed was 51 (ie. 17 plants x 3 sets). All the three independent sets (each comprising of DNA from 17 plants) gave the same result, confirming the consistency of the MSRD-PCR. To add to this is the fact that even the demethylation status was retained across these replicates and, even if one sample had reverted to methylation, it would have been picked in PCR.

Next we wanted to study whether the epigenetic memory retained in third generation. Hence, progeny plants were grown from the seeds of G2 plants. From each of the three G2 sets (I, II and III), third generation seeds were collected and designated as G3 seeds (Fig. 4). Three sets of G3 plants (G3-I, G3-II and G3-III) were raised for DNA extraction (17 plants from each set). Methylation analysis using MSRD-PCR was repeated in G3 plants on the above eight promoters and this revealed that in three genes, *CEN2, LIG4,* and *RAD51*, the memory was preserved (Table 4; Fig. S8). These three genes belonged to NER, NHEJ and HR pathways, and exhibited transgenerational epigenetic memory in response to three different *Agrobacterium*-derived factors, Vir proteins, T-DNA and oncogenes, respectively.

**_Agrobacterium_ induces DNA strand breaks in the host genome**

The most favoured model for *Agrobacterium* T-DNA integration is through NHEJ (Gelvin 2017; Lacroix and Citovsky 2019). Therefore, DNA strand breaks in the plant genome may be vital for T-DNA integration. To test if *Agrobacterium* could induce host DNA strand breaks, we infected 10 *Arabidopsis* plants in the same way as for expression and methylation analysis, with three different *Agrobacterium* strains, VOT, XXX, and VXX (Table 1) and collected the infected tissue at various time after infection (4 h, 24 h, and 48 h). Nuclei were isolated from infected tissue and approximately 400-500 nuclei were analysed using alkaline comet assay. All the *Agrobacterium* strains induced various comet patterns (Fig. 5) in about 5-10% of the nuclei, 4 h post-infection, suggesting DNA breakage. When all the nuclei were viewed under 20X magnification, those with comet patterns appeared to be smaller than the normal ones (Fig. 5). There was not a single nucleus having this pattern at 24 h and 48 h post-infection as well as in the mock-infected control (Fig. 5). Thus, the mere presence of *Agrobacterium* triggered short-lived DNA strand breaks in *Arabidopsis*.

## DISCUSSION

*Agrobacterium* is an exceptional plant pathogen, widely used as a genetic engineering tool and more recently, used as a carrier for delivering genome editing reagents into plants. This soil bacteria has the unique ability to transfer its own DNA, the T-DNA and some proteins such as VirD2 and VirE2 into the host plant cell (Anderson and Moore 1979; de la Riva 1998; Gelvin 2010). T-DNA randomly integrates into the host genome (Gelvin 2010). To facilitate T-DNA entry and integration, Vir proteins interact with host proteins and modulate many host machinery such as defense (Veena et al. 2003) and DNA repair (Gelvin 2010) pathways. Pathogens are known to induce DNA strand breaks (Song and Bent 2014) and enhance

somatic mutation frequencies in plants (Lucht et al. 2002; Kovalchuk et al. 2003). For example, Kathiria et al. (2010) reported enhanced SHR rates in plants infected with TMV. *Agrobacterium*, in contrast, supressed host somatic mutation rates (Shah et al. 2015). Mutations are ultimately controlled by DNA repair genes. There are no previous reports focussing on DNA repair in plants under the influence of *Agrobacterium* and its unique factors such as Vir proteins, T-DNA and oncogenes. Many previous studies (Veena et al. 2003, Deeken et al. 2006, Ditt et al. 2006 and Lee et al. 2009) focused only on the gene expression of *Agrobacterium*-infected tissues and there are no gene expression studies on the effect of *Agrobacterium* infection on the distal tissues. Pathogen infection triggers systemic response in plants and hence distal cells also respond to infection (Deleris et al. 2016; Heil and Ton 2008). Further, *Agrobacterium* being a motile bacterium (Tomlinson and Fuqua 2009), we wanted to study whether the host DNA repair gene expressions responded to the *Agrobacterium* per se or to its unique factors in distal tissue. Previous reports convey that DNA repair genes of HR and NHEJ pathways are involved in T-DNA integration, but there are unknown pathways involved because T-DNA integration took place in plants even when the key genes of HR and/or NHEJ were mutated (Deleris et al. 2016; Heil and Ton 2008). Hence, we studied the expression and promoter methylation of genes belonging to five DNA repair pathways, BER, NER, MMR, HR, and NHEJ. We also wanted to see if these responses were temporal and hence we performed all our experiments at three different time intervals, 4 h, 24 h and 48 h. Since there were previous reports on transgenerational epigenetic changes induced by other pathogens such as *Pseudomonas syringae* pv. *tomato* (PstDC3000) and TMV (Boyko et al. 2007), and the fact that we noticed changes in the methylation profile of DNA repair gene promoters due to *Agrobacterium*, we extended our analysis to the progeny generations as well. We tracked the methylation status of host DNA repair genes for two generations that were not infected by *Agrobacterium*. Apart from the above experiments, we studied whether *Agrobacterium* or its factors induced DNA strand breaks in the host genome as genome stability is associated with DNA repair.

Previous reports on the gene expression profiling in plants upon *Agrobacterium* infection include that of Veena et al. (2003), Deeken et al. (2006), Ditt et al. (2006) and Lee et al. (2009). They performed suppressive subtractive hybridization (Veena et al. 2003) and array-based analyses and, discovered altered expression of genes involved in processes other than DNA repair such as cell division, growth processes (Veena et al. 2003), carbohydrate metabolism, photosynthesis, mitochondrial electron transport, cell wall formation, lipid

metabolism, N-metabolism (Deeken et al. 2006), cell organization and biogenesis, protein metabolism, electron transport or energy pathways (Ditt et al. 2006) and plant defense response (Veena et al. 2003; Lee et al. 2009). Further, Veena et al. (2003) and Ditt et al. (2006) performed experiments on the cell cultures of tobacco and *Arabidopsis*, respectively, and not the whole plant. Deeken et al. (2006) and Ditt et al. (2006) used oncogenic strain of *Agrobacterium* and hence the host response due to Vir proteins and T-DNA could not be distinguished. None of the previous reports used strain lacking oncogenes, *Vir* genes, and T-DNA. By involving such a strain along with wild-type and its other derivatives for our analysis, we could distinguish the host response that was specific to mere presence of bacteria, Vir proteins, oncogenes, and T-DNA.

Our study suggests that presence of T-DNA triggered the expression of genes involved in NER (*CEN2*), HR (*RAD51* and *BRCA2*), and NHEJ (*KU70* and *KU80*), and not BER and MMR. There are previous reports of HR (*RAD51;* van Attikum and Hooykaas 2003) and NHEJ (*KU70* and *KU80*; van Attikum et al. 2001; Friesner and Britt 2003; Li et al. 2005) genes being involved in *Agrobacterium*-mediated host transformation. In support to our observation, in the previous report by Park et al. (2015), the BER gene (*XRCC1*) and HR gene (*ATM*) did not alter T-DNA integration. Our results indicate that these two genes did not alter the T-DNA integration probably because *XRCC1* seems to be under the influence of Vir proteins and not T-DNA and, the expression of *ATM* was not altered by any of the infection conditions. Nevertheless, apart from T-DNA there could be a possibility that other *Agrobacterium* factors have an indirect influence on T-DNA integration. For example, while absence of an NER gene (*XPF*) in *XPF*-deficient *Arabidopsis* mutants led to a small decrease in transformation in a previous report by Mestiri et al. (2014), the expression of *XPF* was increased in the presence oncogenes and not T-DNA in our experiments. The reason for this is not known as there are no previous reports suggesting any correlation between oncogenes and T-DNA integration. Our analysis also conveys the fact that some of the host DNA repair genes can be influenced even by the mere presence of bacterium, independent of T-DNA integration.

Previous reports on pathogen-induced changes in *Arabiodopsis* transcriptome that reveal the DNA repair gene expression profile include that by *Cabbage leaf curl virus* (CaLCuV) (Ascencio-Ibáñez et al. 2008), *Botrytis cinerea*, *P. syringae*, *Phytophthora infestans* and *Erysiphe oronti* (data obtained from electronic fluorescent pictograph (eFP) browser; Winter

et al. 2007). All these pathogens also altered the expression of many DNA repair genes. The expression response of these genes was not similar; the same host (*A. thaliana*) displayed a pathogen-specific response with respect to DNA repair gene expression. For example, while the expression of *RAD23C* was reduced by all the above five pathogens as well as all the *Agrobacterium* strains, the expression of *RAD23B* was reduced by all *Agrobacterium* strains and *Botrytis cinerea*, but not others. There are previous reports of DNA repair genes of HR pathway [*BRCA2* and *RAD51* (Wang et al. 2010), *SSN2* and *RAD51D* (Song et al. 2011; Durrant et al. 2007), *RAD17* and *ATR* (Yan et al. 2013)] regulating defence genes involved in systemic acquired resistance. The absence of the same genes [*BRCA2, RAD51* (Wang et al. 2010), *SSN2* (Song et al. 2011), *RAD51D* (Durrant et al. 2007), *RAD17* and *ATR* (Yan et al. 2013)] in *Arabidopsis* mutants deficient of the respective genes, rendered the plants susceptible to bacterial pathogen *Pseudomonas syringae* pv. *maculicola*. These reports and our work suggest that it is important to investigate more on pathogen-induced changes in host DNA repair system in plants, especially crop plants, in order to improve crops with better immunity. With respect to studies on plant-*Agrobacterium* interaction, we recommend that whole transcriptome analysis be performed after infecting with various strains so that influence of unique bacterial factor can be studied. Though we have not done this analysis, our temporal approach on a sub-set of 22 genes indicates that 24 h can be the chosen post-infection period.

DNA methylation and demethylation are important modes of transcriptional regulation (Finnegan et al. 1998). Biotic and abiotic stresses are known to induce alterations in the methylation status of genomes (Deleris et al. 2016). While our work focussed on DNA repair genes, previous reports of biotic stress-induced differential methylation were on resistance/defense genes, whole genome or transposable elements (Deleris et al. 2016; Hewezi et al. 2018). Differential methylation of genomic regions was induced in the root nodules of *Medicago truncatula* by *Rhizobium*, a close relative of *Agrobacterium* (Satgé et al. 2016). *Arabidopsis* plants infected with *Hyaloperonospora arabidopsidis* (Sánchez et al. 2016) or the cyst nematode *Heterodera schachtii* (Hewezi et al. 2017) and tobacco plants infected with TMV (Wada et al. 2004; Boyko et al. 2007), showed large-scale changes in DNA methylation and these changes were associated with transcriptional changes of defense/stress-related genes. There are also reports on differential methylation of transposable elements in *Arabidopsis* plants subjected to *P. syringae* (Dowen et al. 2012) and *Fusarium oxysporum* (Le et al. 2014).

Methylation in plants is highly dynamic in nature as it constantly changes with plant growth and development as well as in response to environmental stresses (Bartels et al. 2018). Temporal methylation studies were performed previously in plants such as maize under lead stress (Ding et al. 2014) and wounding (Lewandowska-Gnatowska et al. 2014), and *Arabidopsis* after *P. syringae* infection (Pavet et al. 2006). Previous report on *Agrobacterium*-induced temporal changes in DNA methylation includes global methylation/demethylation analyzed in soybean at 0-90 min and 6-72 h post-infection (Jiang et al. 2016). The global methylation level was measured using HPLC based method and therefore the sequence of the methylated/demethylated region due to the influence of *Agrobacterium* was not reported.

There are a number of reports on the epigenetic processes associated with crown gall development, oncogene expression, and T-DNA integration (Gohlke et al. 2014). For example, global hypermethylation and promoter hypomethylation were observed in crown gall genome (Gohlke et al. 2013). Because this study involved only tumorigenic *Agrobacterium* strain, responses specific to Vir proteins, T-DNA or PAMP were undistinguishable. Our experiments involved 16 different treatment conditions, which included the controls and infections by four different strains under three different time intervals. This study not only revealed the influence the various bacterial factors on the methylation status of DNA repair genes, but also displayed the dynamics of methylation. It was interesting to observe that each of *Agrobacterium*-derived factors induced a specific methylation profile in 14 out of 22 host DNA repair gene promoters analysed. Other examples of epigenetic modifications due to *Agrobacterium* include those associated with T-DNA insertion sites (Gelvin et al. 1983; Hepburn et al. 1983) and transgene either after (Jupe et al. 2019; Linne et al. 1990; Kilby et al. 1992) or before (Philips et al. 2019) integration.

Alteration of methylation status of promoters often leads to change in gene expression and methylation mostly leads to reduced transcription (Zhang et al. 2018; Woo et al. 2007). In our study, only two genes (*KU70* and *RAD23C*) showed this expected correlation. In contrast, there are reports suggesting that there could be situations when methylation enhance gene expression (Harris et al. 2018; Zhang et al. 2018) and two genes (*RAD4* and *RAD51*) probably followed this trend. Surprisingly, all the remaining genes analysed, which is the majority, did not exhibit any correlation between expression and promoter methylation. In these cases, expression and methylation was influenced by different factors. This non-correlation could be due to the influence of some other regulatory mechanism (example those involving histone modifications, miRNA or transcription factors), which needs further investigation. Though we

have selected multiple MSREs with many sites in the promoters for digestion, there is also a possibility that some other regions could be methylated/demethylated that regulate gene expression. There are also possibilities of spreading of methylation into the promoter from neighbouring regions (Zhang et al. 2018). Also, it is to be noted that though some of the epigenetic signatures remained in the plant for multiple generations (Fig. S10, discussed below), their expression returned to normal by 48 h post infection. Hence, it is yet to be understood what these epigenetic signatures mean.

DNA methylation/demethylation especially, cytosine methylation, is the epigenetic mark that can faithfully pass on to the progeny generations (Mathieu et al. 2007). There are very few reports on plant-microbe interaction studies involving epigenetic memory of host DNA in terms of DNA methylation. For example, Boyko et al. (2007) reported that the progeny of tobacco plants infected with TMV exhibited hypomethylation of several LRR-containing loci. TMV also lead to hypermethylation of the progeny genome and (Boyko et al. 2007; Kathiria et al. 2010). In contrast, reports by Luna et al. (2012) indicate that PstDC3000 induced hypomethylation of *Arabidopsis* genome in infected parent as well as the uninfected progeny of the infected parent. The epigenetic memory in the immediate progeny of the treated parent is termed as intergenerational memory and that in subsequent generations is termed as transgenerational memory (Heard and Martienssen 2014; Lämke and Bäurle 2017). There are no previous reports on intergenerational/transgenerational epigenetic memory of *Agrobacterium* infection in plants. Of the 14 gene promoters that had altered methylation status in first generation, five exhibited intergenerational memory as it lasted only up to second generation (Fig. S8). In three out of 14 promoters, the memory lasted up to third generation (Fig. S8), setting an example of transgenerational memory. In order get a detailed understanding of the methylation status, we generated an epigenetic profile of each promoter (Fig. S10). This revealed that not all epigenetic signatures in the same promoter were heritable. For example, in *RAD23D* promoter, demethylation was seen to be retained in progeny only upon digestion with *Hpy*ChIV4 and not *Bsa*AI (Fig. S10). Also, when it comes to cases of multiple sites for the same enzyme in the same promoters, as in *RAD23D* again (Fig. S10), where there were two *Hpy*ChIV4 sites, we do not know whether both or any one of the site was demethylated because in either case the DNA would be digested with *Hpy*ChIV4 and we could get the same PCR result. Nevertheless, our approach of studying the dynamic influence of multiple strains in a sub-set of 22 DNA repair genes has confirmed that the *Agrobacterium* does induce transgenerational epigenetic memory in the host and this

memory is very specific to the unique factors of *Agrobacterium*. Considering the above-mentioned short comings of MSRD-PCR and the fact that this method cannot verify the entire promoter sequence or the neighbouring influential sequences in the chromosome, we recommend whole epigenome analysis inclusive of adopting our approach of infecting with multiple strains.

Studies on epigenetic memory could possibly elucidate adaptation strategies of plants to biotic or abiotic stresses (Baulcombe and Dean 2014; Zhang et al. 2018). For example, in a previous study by Luna et al. (2012) progeny of the parent infected with PstDC3000 exhibited resistance against PstDC3000 and *Hyaloperonospora arabidopsidis* and, were susceptible to *Alternaria brassicicola*. Progeny of tobacco plants infected with TMV had higher levels of *pathogenesis-related gene1* expression and exhibited delayed symptom development when subjected to infection with either TMV, *P. syringae*, or *Phytophthora nicotianae* (Kathiria et al. 2010). Apart from adaptation, there are also instances where pathogen stress in plants enhanced somatic mutations in the infected parent as well the uninfected progeny. TMV infection of tobacco plants lead to enhanced SHR in infected parents as well as the uninfected progeny (Boyko et al. 2007; Kathiria et al. 2010). Like any other plant pathogen, *Agrobacterium* is also a biotic stress and we do not know what adaptations it could induce in the host. Hence, our results has opened questions as to whether the moto of epigenetic changes could be resistance or susceptibility to other pathogens or *Agrobacterium* or even T-DNA integration. It is to be noted that only 5% of total gene promoters in *Arabidopsis* are regulated by methylation (Zhang et al. 2006; Zhang et al. 2018). In our study, about 63% of DNA repair genes analysed showed altered methylation in the parental generation. Of these about 57% exhibited epigenetic memory as well. Interestingly, two genes, *RAD51* and *KU70*, belonging to HR and NHEJ pathways respectively, underwent enhanced expression in parental generation and exhibited epigenetic memory in progeny generation in response to T-DNA. The purpose of this epigenetic change and intriguing memory exhibited by other DNA repair genes, in response to specific *Agrobacterium* factors, is yet to be understood.

In addition, all the previous studies looked at changes in gene expression in the infected cells and we focused on gene expression and analysis of methylation status in uninfected distal cells. The fact that many genes displayed altered methylation in DNA extracted from tissue far away from infection site and not mock-infected sites, our results are an indication that there is some information, specific to the bacteria and its unique factors, being passed on from

the site of infection to the distal regions. Further, inflorescence originated from the distal cells and the altered methylation was observed in progeny also. The nature of the transmissible information is not known. However, there could be two possibilities, one, the unknown information travelled systemically and two, only selective distal cells received it. Presence of amplification in MSRD-PCR would mask the response of distal cells that did not receive the signal. Towards this end, we would like to bring to notice that we had obtained many examples of demethylation also where, the DNA from the control plants gave amplification while that from the infected plant did not. Absolute absence of amplification, which is the indication of demethylation, was possible only if the distal cells responded uniformly. This could happen only if the unknown signal transmitted systemically and not to selective cells. In nature also, *Agrobacterium* infects crown part of stem and signal travels to upper part of the plant.

It is known that genome stability of any organism is much dependent on its DNA repair system. Also, it was previously proposed that DNA strand breaks are induced in to the host genome by *Agrobacterium*, prior to T-DNA integration (Gelvin 2017; Lacroix and Citovsky 2019). However, there was no report showing DNA strand breaks in plants after *Agrobacterium* infection. Since we observed that many DNA repair genes, even those which were not reported to be involved in the process of T-DNA integration were affected by *Agrobacterium*, we analysed the host genome stability in terms of DNA strand breaks. We did observe short-lived *Agrobacterium*-induced DNA strand breaks in *Arabidopsis* genome at 4 h only and not at 12 h or 24 h post-infection. *Agrobacterium* PAMPs could be the probable reason for the breaks as these were induced by all the strains irrespective of their unique factors and not by mock infection. Other pathogens are also known to induce DNA strand breaks in the host genome. Song and Bent (2014) and Cerovska et al. (2014) reported that virulent bacteria *P. syringae* pv. *tomato* induced double strand breaks (DSBs) in *Arabidopsis* and *Potato virus X* induced DNA strand breaks in *N. tabacum*, respectively. Similar to plants, there are reports of pathogens inducing DNA damage in animals as well. For example, *Escherichia coli* induced DSB breaks in animal epithelial cells (Nougayrède et al. 2006). Reversion of DNA damage, like what we observed, was not reported in these earlier reports. DNA repair system is much conserved across the higher eukaryotic system, including plants and animals (Gimenez and Manzano-Agugliaro 2017). Similar to our report, repair of DSB was observed in mammalian cells 24 h after they were subjected to ionizing radiation (Riballo et al. 2004; Löbrich et al. 2010). It could be possible that in a similar manner, the DNA repair

machinery of *Arabidopsis* was also efficient to repair the damage within a period of 24 h. Our work reveals that T-DNA is not at all a requisite for whole genome DNA breaks and that mere presence of *Agrobacterium* is sufficient to trigger this response. This indicates that plants respond to *Agrobacterium* just like the way they do to other pathogens as mentioned above, in terms of DNA damage and, it could be just that the T-DNA gets trapped in the genome during the process of DNA repair. However, T-DNA integration takes place beyond 24 h of infection as well and may involve other methods such as microhomology-based DNA repair (Gelvin 2017; Lacroix and Citovsky 2019; van Kregten et al. 2016) or other unknown method. Considering these observations and the fact that many DNA repair genes from NER, HR and NHEJ pathways were upregulated due to T-DNA, it could be possible that T-DNA integration may not rely on any single method and can take place in multiple parallel ways.

## CONCLUSIONS

This study was aimed at understanding the plant DNA repair machinery under the influence of *A. tumefaciens*, the natural genetic engineer, and its unique factors (Vir proteins, T-DNA and oncogenes). Since the temporal analysis of gene expression revealed that most of the DNA repair genes had altered expression 24 h post-infection with *Agrobacterium*, which stabilized by 48 h post-infection, we recommend 24 h post-infection as the best time for studying plant DNA repair system upon *Agrobacterium* infection. Our approach of performing experiments using four different *Agrobacterium* strains revealed for the first time that the DNA repair gene expression as well as promoter methylation was very specific either to Vir proteins, T-DNA, oncogenes or mere presence of bacteria (PAMP). Our results of gene expression and methylation studies in tissues distal from the infection sites indicate that there is some unknown information, specific to various *Agrobacterium*-derive factors, spreading across the plant from the site of infection. Promoters of at least three genes, *CEN2, RAD51,* and *LIG4*, belonging to NER, HR, and NHEJ pathways, respectively, exhibited epigenetic memory specific to one of the bacterial factors, up to three generations. Further, *Agrobacterium*, irrespective of the presence or absence of its unique factors, induced temporary whole genome DNA strand breaks in *Arabidopsis*. Our results of DNA repair gene expression and DNA strand breaks and, previous reports on microhomology-based T-DNA integration, when considered together, it indicates that T-DNA integration may take place in multiple parallel ways involving random breaking of host DNA or microhomology or any other unknown method. Thorough understanding the DNA repair mechanism during

*Agrobacterium* infection may help to develop new strategies for plant transformation, especially in plants recalcitrant to T-DNA integration. Moreover, the transgenerational epigenetic memory of *Agrobacterium* infection points towards a possibility of priming plants using disarmed *Agrobacterium* strain in order to enhance resistance against some pathogens and/or enhance *Agrobacterium*-mediated plant transformation rates so as to achieve efficient genome editing and/or genetic engineering.


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## Figure captions

**Fig. 1** Methylation-sensitive restriction digestion (MSRD)-PCR. **a** Restriction map obtained using NEB cutter for *CEN2* gene promoter region; the two arrows facing each other indicate primers and the numbers between these arrows indicate primer location and the MSRE sites circled are the ones whose methylation status will be studied using MSRD-PCR. Similar restriction maps for rest of the genes are in Fig. S1. **b** An outline of MSRD-PCR. The horizontal thick line indicates DNA; the bulb-like projections indicate methylation; MSRE is a methylation-sensitive restriction enzyme; arrows are locations of forward and reverse primers. **c** gel image of MSRD-PCR. The lanes L, T1, T2, T3, and T4 indicate 100-bp ladder, treatment 1, treatment 2, treatment 3 and treatment 4 respectively. The areas circled in red indicate T2 and T4, the treatment conditions during which promoter region was methylated.

**Fig. 2** Heat map of expression profiles of DNA repair genes under different treatment conditions. Mock-infected plants served as the controls. The scale shows $\log_2$ fold change. Statistical significance of expression is represented in Fig. S5. The experiment was done independently three times.

**Fig. 3** Gel images of methylation-sensitive restriction digestion-PCR (MSRD-PCR) showing different methylation patterns exhibited by DNA repair gene promoters in response to Vir proteins, T-DNA, oncoproteins, presence of bacteria (probably due to PAMP) and wounding. The genes were classified as methylated or demethylated by comparing with uninfected control (C). The respective methylation-sensitive restriction enzyme used for digestion is given at the right side of each gel image. L, C, M, U and W indicate 100-bp ladder, uninfected control, mock-infected, undigested DNA and water control. The numbers 4, 24, and 48

indicates three different time intervals in hours. Note that only representative gel images have been shown in this figure and additional images are in category G1 of Fig. S8.

**Fig. 4** A detailed outline of the epigenetic study per treatment. G1, G2, and G3 indicate 3 successive generations. 'n' is the number of plants taken in each step.

**Fig. 5** Host DNA strand break induced by various *Agrobacterium* strains. Three different comet patterns obtained after 4 hours of infection and no visible damage at 24 and 48 h post-infection, at 40 X magnification.

**Table 1** Various *Agrobacterium* strains used and their details

| Name of the strain | Symbol | Description | Bacterial factors‡ | | | Antibiotics used (10 mg/l) | Reference |
|---|---|---|---|---|---|---|---|
| | | | T-DNA | Vir proteins | Oncogenes | | |
| Ach5 | VOT | Wild type | + | + | + | Rifampicin | Shah et al. 2015 |
| LBA4002 | XXX | Avirulent, non tumerigenic | - | - | - | Rifampicin | |
| LBA4404 | VXX | Virulent, non tumerigenic | - | + | - | Rifampicin | |
| LBA4404 (pCAMBIA2300) | VXT | Virulent, non tumerigenic | + | + | - | Rifampicin and Kanamycin | |
| LBA4404 (pCAMBIA2301)† | Not applicable | *GUS*-positive strain of VXT | + | + | - | Rifampicin and Kanamycin | |

† LBA4404 (pCAMBIA2301) was used for confirming infection and not used for expression and other analyses.

‡ + and – denotes presence and absence, respectively.

**Table 2** Methylation pattern† in infected plants at different infection conditions

| Type of repair | Gene name | Methylation status‡ | | | | | | | | | | | | | | | | Methylation/ demethylation | Probable factor |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Control | Mock | | | VOT | | | XXX | | | VXT | | | VXX | | | | |
| | | | 4 | 24 | 48 | 4 | 24 | 48 | 4 | 24 | 48 | 4 | 24 | 48 | 4 | 24 | 48 | | |
| NER | *RAD23C* | ✓ | - | - | - | - | - | ✓ | - | - | ✓ | - | - | ✓ | - | - | ✓ | Demethylation<br>Methylation | Mock inoculation<br>Presence of bacteria |
| | *RAD23D* | ✓ | ✓ | ✓ | ✓ | - | - | - | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | Demethylation | Oncogenes |
| | *RAD23B* | ✓ | ✓ | ✓ | ✓ | - | - | - | ✓ | ✓ | ✓ | - | - | - | - | - | - | Demethylation | Vir proteins |
| | *XAB2* | - | - | - | - | ✓ | ✓ | - | - | - | - | ✓ | ✓ | - | ✓ | ✓ | - | Methylation | Vir proteins |
| | *RAD4* | - | - | - | - | ✓ | ✓ | - | | - | - | ✓ | ✓ | - | ✓ | ✓ | - | Methylation | Vir proteins |
| | *CEN2* | - | - | - | - | - | ✓ | ✓ | - | - | - | - | ✓ | ✓ | - | ✓ | ✓ | Methylation | Vir proteins |
| | *XPF* | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | - | - |
| BER | *MBD4* | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| | *TAG1* | - | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | Methylation | Mock inoculation |
| | *P3MAG1* | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| | *XRCC1* | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| HR | *RAD51* | - | - | - | - | - | ✓ | ✓ | - | - | - | - | ✓ | ✓ | - | - | - | Methylation | T-DNA |
| | *BRCA2* | - | - | - | - | - | ✓ | ✓ | - | - | - | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | Methylation | Vir proteins |
| | *ATM* | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| | *BLM* | - | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | - | Methylation | Mock inoculation |
| | *MRE11A* | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | - | - |
| MMR | *PMS2L3* | - | - | - | - | ✓ | ✓ | ✓ | - | - | - | - | - | ✓ | - | - | ✓ | Methylation | Vir proteins and oncogenes |
| | *MSH4* | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| | *MSH6* | ✓ | ✓ | ✓ | ✓ | - | - | ✓ | - | - | - | - | - | - | - | - | - | Demethylation | Presence of bacteria |
| NHEJ | *KU70* | ✓ | ✓ | ✓ | ✓ | - | - | - | ✓ | ✓ | ✓ | - | - | - | ✓ | ✓ | ✓ | Demethylation | T-DNA |
| | *KU80* | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| | *LIG4* | - | - | - | - | - | - | ✓ | - | - | - | - | - | - | - | - | - | Methylation | Oncogenes |

NER, nucleotide excision repair; BER, base excision repair; MMR, mismatch repair; HR, homologous recombination; NHEJ, non-homologous end joining.

† Methylation response against individual methylation-sensitive restriction enzymes is given separately in Table S3.

‡✓ and – denotes presence and absence of methylation, respectively.

**Table 3** Comparing the influence of various infection factors on the expression and methylation of DNA repair genes

| Type of repair | Gene name | Expression | Affecting factor | Methylation/ demethylation | Affecting factor |
|---|---|---|---|---|---|
| NER | *RAD23C* | Down | Presence of bacteria | Demethylation | Mock inoculation |
| | | | | Methylation | Presence of bacteria |
| | *RAD23D* | Down | Presence of bacteria | Demethylation | Oncoproteins |
| | *RAD23B* | Down | Presence of bacteria | Demethylation | Vir proteins |
| | *XAB2* | Down | Presence of bacteria | Methylation | Vir proteins |
| | *RAD4* | Up | Vir proteins | Methylation | Vir proteins |
| | *CEN2* | Up | T-DNA | Methylation | Vir proteins |
| | *XPF* | Up | Oncoproteins | No change | NA |
| BER | *MBD4* | Down | Vir proteins | No change | NA |
| | *TAG1* | No change | NA | Methylation | Mock inoculation |
| | *P3MAG1* | Up | Presence of bacteria | No change | NA |
| | *XRCC1* | Up | Vir proteins | No change | NA |
| HR | *RAD51* | Up | T-DNA | Methylation | T-DNA |
| | *BRCA2* | Up | T-DNA | Methylation | Vir proteins |
| | *ATM* | No change | NA | No change | NA |
| | *BLM* | Down | Vir proteins | Methylation | Mock inoculation |
| | *MRE11A* | Up | Vir proteins | No change | NA |
| MMR | *PMS2L3* | No change | NA | Methylation | Vir proteins and oncoproteins |
| | *MSH4* | No change | NA | No change | NA |
| | *MSH6* | Down | Vir proteins | Demethylation | Presence of bacteria |
| NHEJ | *KU70* | Up | T-DNA | Demethylation | T-DNA |
| | *KU80* | Up | T-DNA | No change | NA |
| | *LIG4* | Up | Vir proteins | Methylation | Oncoproteins |

**Table 4** Consolidated methylation/demethylation status of DNA repair promoters that exhibited epigenetic memory

| Type of repair | Gene name | Generations | Methylation status | | | | | | Methylation status under analysis | Epigenetic memory-inducing factor |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | Control | Mock | VOT | XXX | VXT | VXX | | |
| NER | *RAD23C* | I | ✓ | - | ✓ | ✓ | ✓ | ✓ | Demethylation | Mock inoculation |
| | | II | ✓ | - | ✓ | ✓ | ✓ | ✓ | | |
| | | III | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | | |
| | *RAD23D* | I | ✓ | ✓ | - | ✓ | ✓ | ✓ | Demethylation | Oncogenes |
| | | II | ✓ | ✓ | - | ✓ | ✓ | ✓ | | |
| | | III | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | | |
| | *RAD23B* | I | ✓ | ✓ | - | ✓ | - | - | Demethylation | No memory |
| | | II | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | | |
| | | III | NA | | | | | | NA | |
| | *XAB2* | I | - | - | ✓ | - | ✓ | ✓ | Methylation | |
| | | II | - | - | - | - | - | - | | |
| | | III | NA | | | | | | NA | |
| | *RAD4* | I | - | - | ✓ | - | ✓ | ✓ | Methylation | |
| | | II | - | - | - | - | - | - | | |
| | | III | NA | | | | | | NA | |
| | *CEN2* | I | - | - | ✓ | - | ✓ | ✓ | Methylation | Vir proteins |
| | | II | - | - | ✓ | - | ✓ | ✓ | | |
| | | III | - | - | ✓ | - | ✓ | ✓ | | |
| BER | *TAG1* | I | - | ✓ | ✓ | ✓ | ✓ | ✓ | Methylation | No memory |
| | | II | - | - | - | - | - | - | | |
| | | III | NA | | | | | | NA | |
| HR | *BLM* | I | - | ✓ | ✓ | ✓ | ✓ | ✓ | Methylation | Mock inoculation |
| | | II | - | ✓ | ✓ | ✓ | ✓ | ✓ | | |
| | | III | - | - | - | - | - | - | | |
| | *BRCA2* | I | - | - | ✓ | - | ✓ | ✓ | Methylation | Vir proteins |
| | | II | - | - | ✓ | - | ✓ | ✓ | | |
| | | III | - | - | - | - | - | - | | |
| | *RAD51* | I | - | - | ✓ | - | ✓ | - | Methylation | T-DNA |
| | | II | - | - | ✓ | - | ✓ | - | | |
| | | III | - | - | ✓ | - | ✓ | - | | |
| MMR | *PMS2L3* | I | - | - | ✓ | - | ✓ | ✓ | Methylation | No memory |
| | | II | - | - | - | - | - | - | | |
| | | III | NA | | | | | | NA | |
| | *MSH6* | I | ✓ | ✓ | - | - | - | - | Demethylation | |
| | | II | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | | |
| | | III | NA | | | | | | NA | |
| NHEJ | *KU70* | I | ✓ | ✓ | - | ✓ | - | ✓ | Demethylation | T-DNA |
| | | II | ✓ | ✓ | - | ✓ | - | ✓ | | |
| | | III | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | | |
| | *LIG4* | I | - | - | ✓ | - | - | - | Methylation | Oncogenes |
| | | II | - | - | ✓ | - | - | - | | |
| | | III | - | - | ✓ | - | - | - | | |

NER, nucleotide excision repair; BER, base excision repair; MMR, mismatch repair; HR, homologous recombination; NHEJ, non-homologous end joining. ✓ and – denotes presence and absence of methylation, respectively. NA is not applicable.

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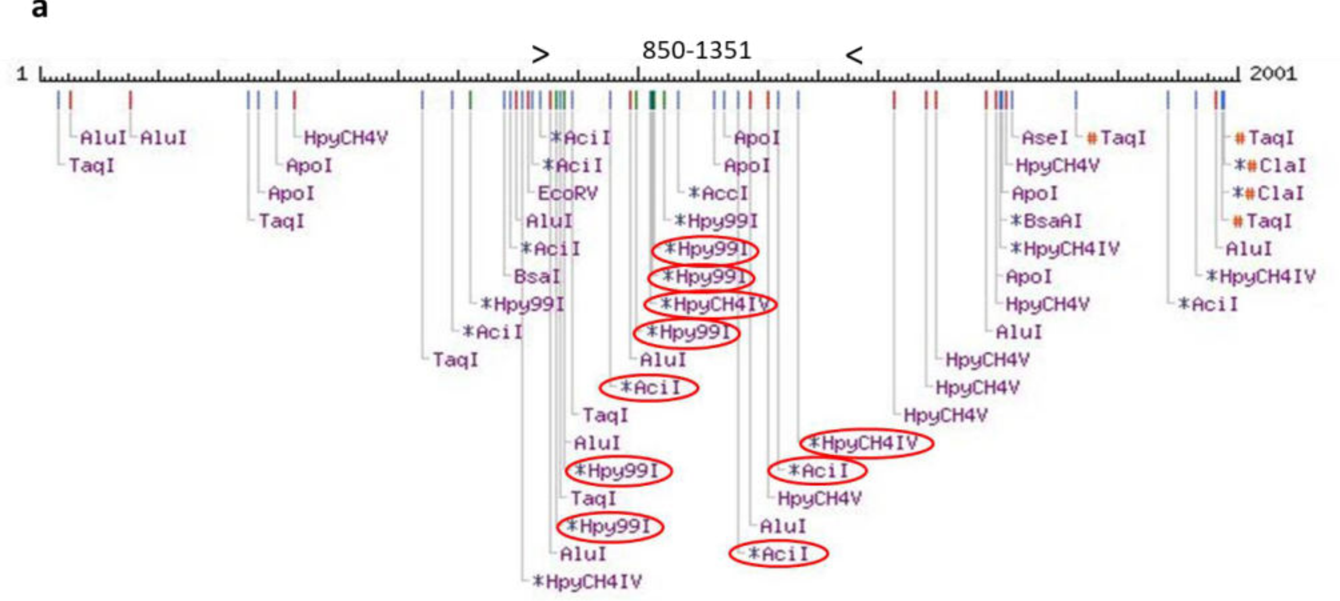
Fig. 1

**a**




**b**

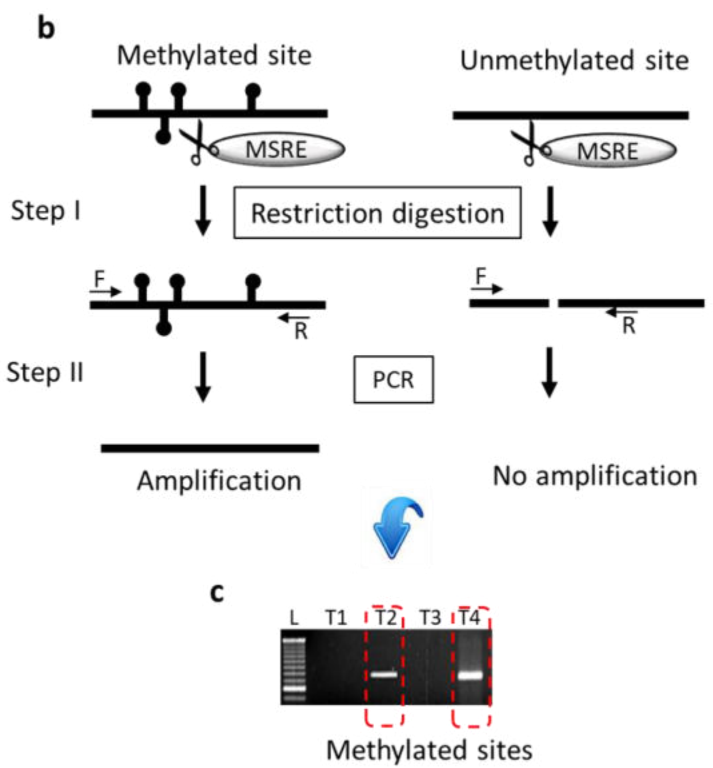


Fig. 2

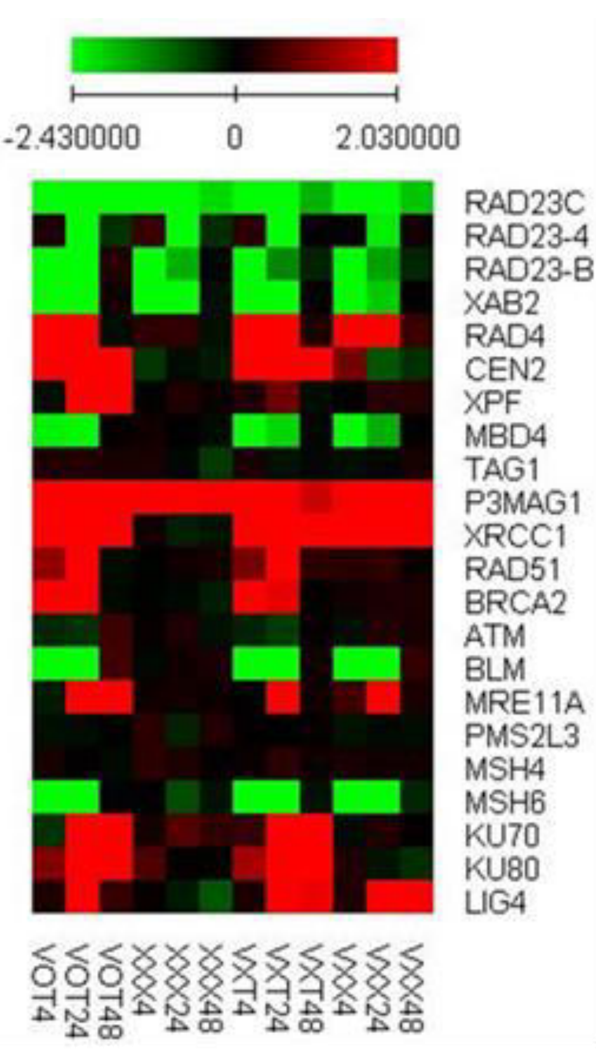


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Fig. 3

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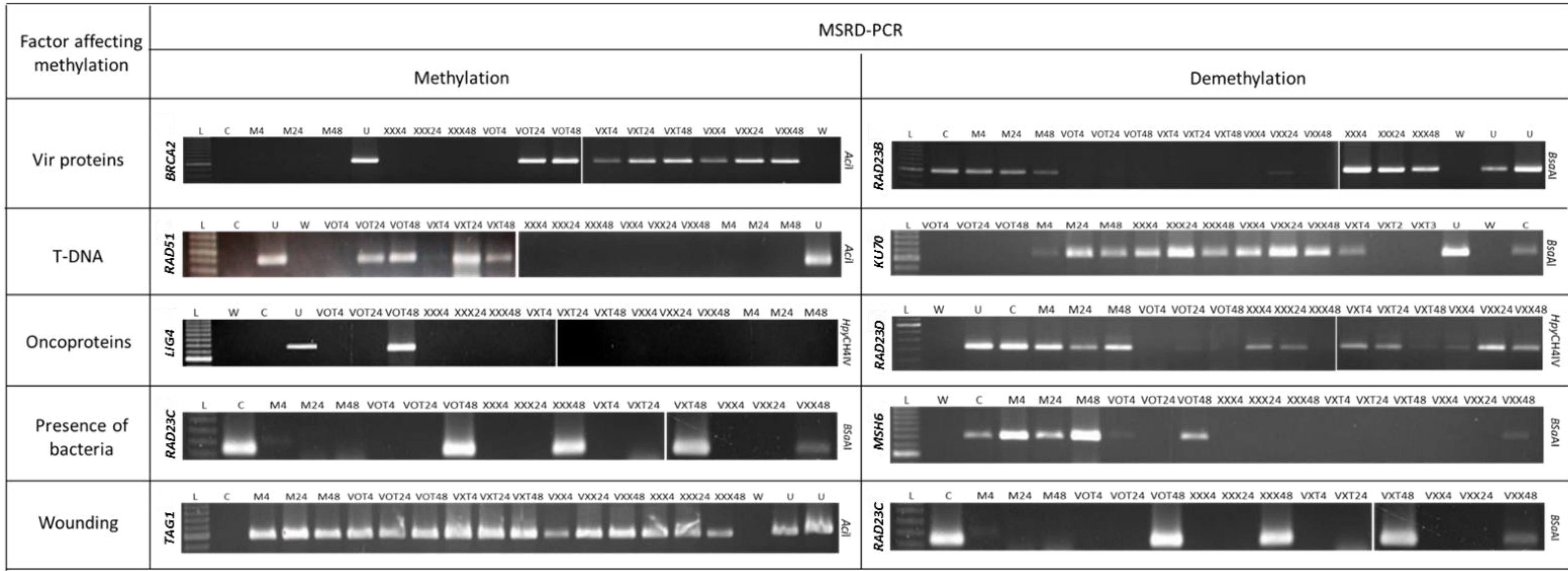


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Fig. 4

1st or parental generation

Agrobacterium

G1 plants
n=20

G1
n=17

Pooled-DNA extraction

Methylation analysis

I II III

Seed collection

G2-I G2-II G2-III

2nd generation

G2-I plants
n=20

G2-II plants
n=20

G2-III plants
n=20

G2
n=17

Pooled-DNA extraction

Methylation analysis

I II III

Seed collection

G3-I

G2
n=17

Pooled-DNA extraction

Methylation analysis

I II III

Seed collection

G3-II

G2
n=17

Pooled-DNA extraction

Methylation analysis

I II III

Seed collection

G3-III

3rd generation

G3-I plants

G3
n=17

Pooled-DNA extraction

Methylation analysis

G3-II plants

G3
n=17

Pooled-DNA extraction

Methylation analysis

G3-III plants

G3
n=17

Pooled-DNA extraction

Methylation analysis

Fig. 5

| Treatments | 4 hour | | | 24 hour | 48 hour |
|---|---|---|---|---|---|
| Mock-infected | | | | | |
| VOT | | | | | |
| VXX | | | | | |
| XXX | | | | | |

50 µm

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