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ORIGINAL ARTICLE

Methylation Status of *Arabidopsis* DNA Repair Gene Promoters During *Agrobacterium* Infection Reveals Epigenetic Changes in Three Generations

T. Joyous Joseph[1] · Chandhini S[1] · Swati Das[1,2] · S. Kirankumar Mysore[3] · M. Jasmine Shah[1]

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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 analysed. 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 the 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 short-lived DNA strand breaks in *Arabidopsis* cells, irrespective of the presence or absence of virulence genes and T-DNA.

Keywords DNA repair · Epigenetic memory · Methylation · DNA strand break · *Agrobacterium* · *Arabidopsis*

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 DNA repair genes exhibited transgenerational memory in response to *Agrobacterium*-derived factors.

✉ M. Jasmine Shah
jasmine@cukerala.ac.in

[1] Department of Plant Science, Central University of Kerala, Periye, Kasaragod, India

[2] Division of Applied Life Science, Gyeongsang National University, Jinju, Republic of Korea

[3] Plant Biology Division, The Samuel Roberts Noble Foundation, Ardmore, OK 73401, USA

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. The ability of *Agrobacterium* for inter-kingdom gene transfer is highly valued and utilized. Recently, *Agrobacterium* is also used to transfer genome editing reagents into plants (Sardesai and Subramanyam 2018). Studying the host response to *Agrobacterium* infection has revealed many interesting facts about plant defense and DNA repair. The interactions of CAK2M (cyclin-dependent kinase activating kinase) and TBP (TATA box-binding protein), which represent the components of plant transcriptional and DNA repair machineries, with VirD2, may target the entire T-complex to the host chromatin (Bak´o et al. 2003). The products of plant host genes such as some

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histone-encoding genes (*H2A*, *H3-11*, *H4*) and some DNA repair genes (*MRE11*, *XRS2*, *SIR4*, and *RAD50*) play important roles in transformation (Tenea et al. 2009; van Attikum and Hooykaas 2003; Friesner and Britt 2003). Veena et al. (2003) reported an approach of *Agrobacterium* for the entry and integration of foreign DNA by suppressing host defense. During the integration step, *Agrobacterium* may use host NHEJ DNA repair machinery (van Attikum and Hooykaas 2003; Friesner and Britt 2003). However, later studies by Park et al. (2015) conclusively showed that T-DNA integration does not require the known NHEJ proteins, suggesting an alternative route for integration. Later on, van Kregten et al. (2016) reported that DNA polymerase theta (Polθ) is essential for T-DNA integration in *Arabidopsis*. However, the actual mechanism of T-DNA integration and associated changes occurring in the host genome upon infection are not completely elucidated. 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. Suppressive subtractive hybridization 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 that the expression of defense genes, various histone-encoding genes, and others but not DNA repair genes were altered. 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 focussing 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 focussed 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 were 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 were 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 the expression and methylation patterns could be studied elaborately. Apart from the previously reported NHEJ 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 a detailed 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 4-week-old *Arabidopsis* plants by making wounds (4–5 pricks) using a 25 gauge needle attached to a 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 4-week-old plants. In experiments involving plant epigenetic memory or comet assay, 3-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 was carefully transferred from MS media to vermicompost obtained from CPCRI (Central Plantation Crops Research Institute, Kasaragod, India), 1 week after infection. Seeds collected from these plants (2nd generation) were again grown on MS medium for three weeks and subjected to DNA extraction. Similar to the first generation, 4-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 lx), temperature (24 °C), and humidity (100%) with a 16-h light/8-h dark cycle.

Beta-GUS Histochemical Staining

Infected and mock-infected plants were incubated at 37 °C for 48 h in glucuronidase (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.

Table 1 Various *Agrobacterium* strains used and their details

Name of the strain	Symbol	Description	Bacterial factors‡			Antibiotics used*	Reference
			T-DNA	Vir proteins	Oncogenes		
Ach5	VOT	Wild type	+	+	+	Rifampicin	Shah et al. (2015)
LBA4002	XXX	Avirulent, non tumourigenic	-	-	-	Rifampicin	
LBA4404	VXX	Virulent, non tumourigenic	-	+	-	Rifampicin	
LBA4404 (pCAMBIA2300)	VXT	Virulent, non tumourigenic	+	+	-	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 - denote presence and absence, respectively *Working concentrations of Rifampicin and Kanamycin are 10 mg/l and 100 mg/l, respectively

Selection of *Arabidopsis* DNA Repair Genes, Promoter Region Identification, Primer Design, and 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. (2019). 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).

Standardization 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 s, different primer annealing Tm (Table S1) for 40 s and 72 °C for 40 s 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 pmol/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 s, different primer annealing Tm (Table S1) for 15 s and 72 °C for 15 s. 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. 2019), 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 normalizing the Cp values with mock-infected control and *UBQ10*. 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.

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 the absence of methylation and, in contrast, lack of digestion/

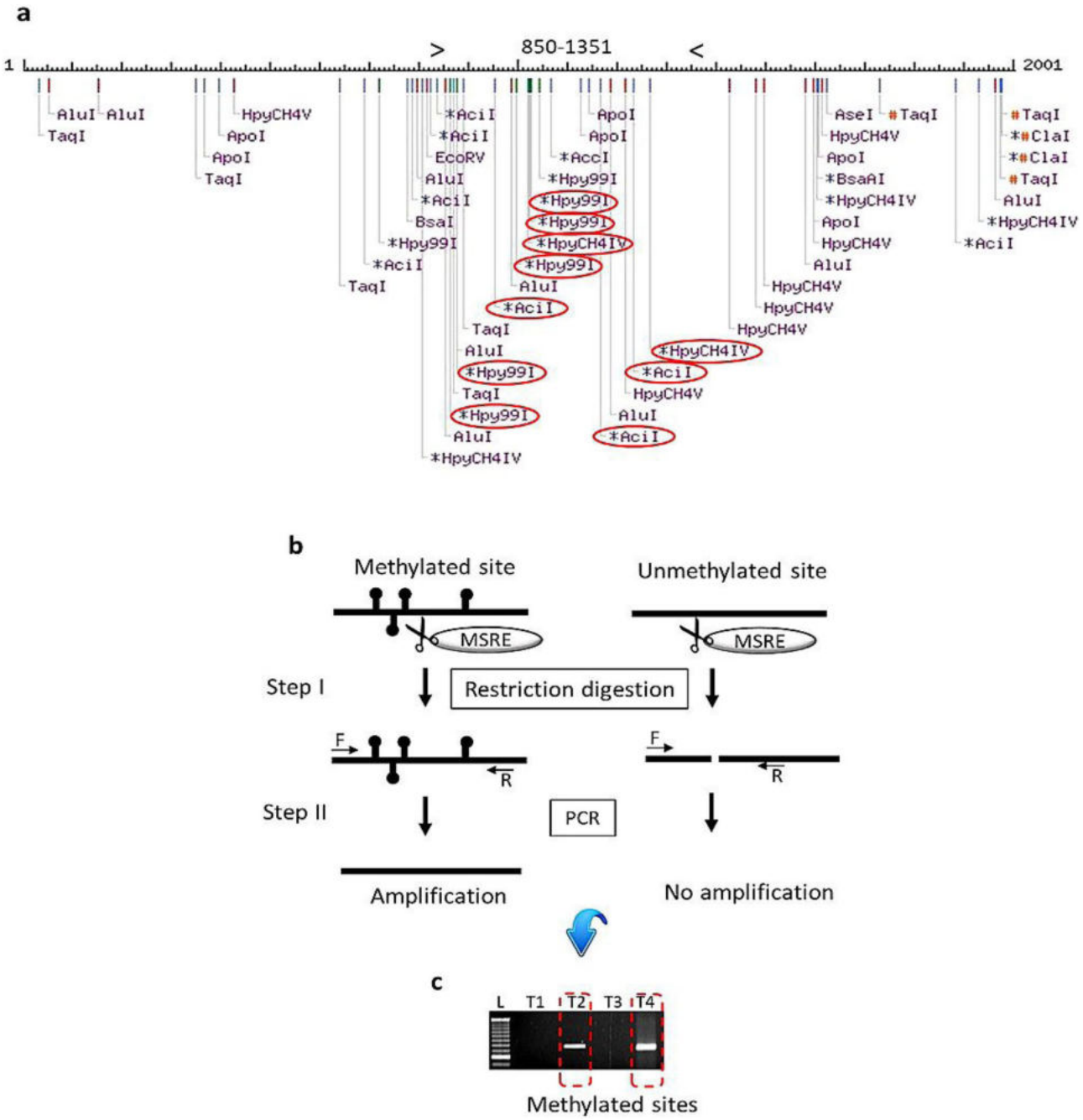

Fig. 1 Methylation-sensitive restriction digestion (MSRD)-PCR. **a** Restriction map obtained using NEB cutter for *CEN2* gene promoter region; the two arrows facing each other indicate primers, and the numbers between these arrows indicate primer location and the MSRE sites circled are the ones whose 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 the 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

incomplete digestion leads to the presence of a band indicating methylation (Fig. 1). The optimum conditions for MSRE digestion were standardized 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 periods (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 concentration 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 2nd (G2-I, G2-II, G2-III) and 3rd (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 Na_2HPO_4, 4 mM NaH_2PO_4, 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–× 40 magnifications.

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 using $2^{-\Delta\Delta Ct}$ value obtained by normalizing the Cp values with mock-infected control and *UBQ10* (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 expressions with respect to mock-infected control were 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

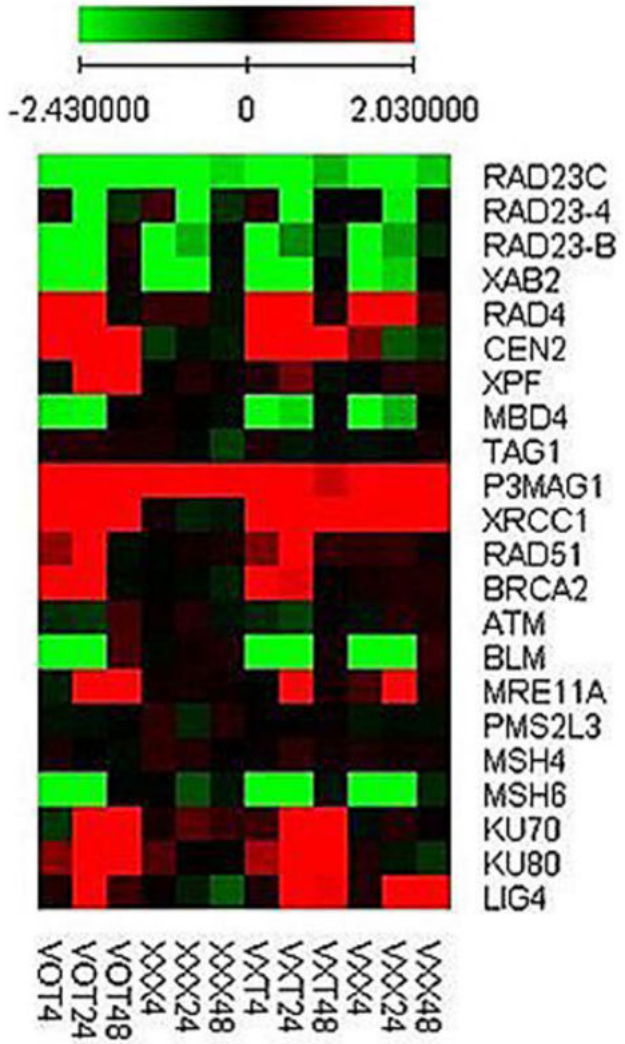

Fig. 2 Heat map of expression profiles of DNA repair genes under different treatment conditions. Mock-infected plants served as the controls. The scale shows $\log_2$ fold change. Statistical significance of expression is represented in Fig. S5. The experiment was done independently three times

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 were 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 at 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 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

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 at different regions of the promoter, consisting of a minimum of four MSRE sites, with the reverse primer binding about 50–1400 bp away from the start codon (Fig. 1; Fig. S1). 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. The presence of a 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 were 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

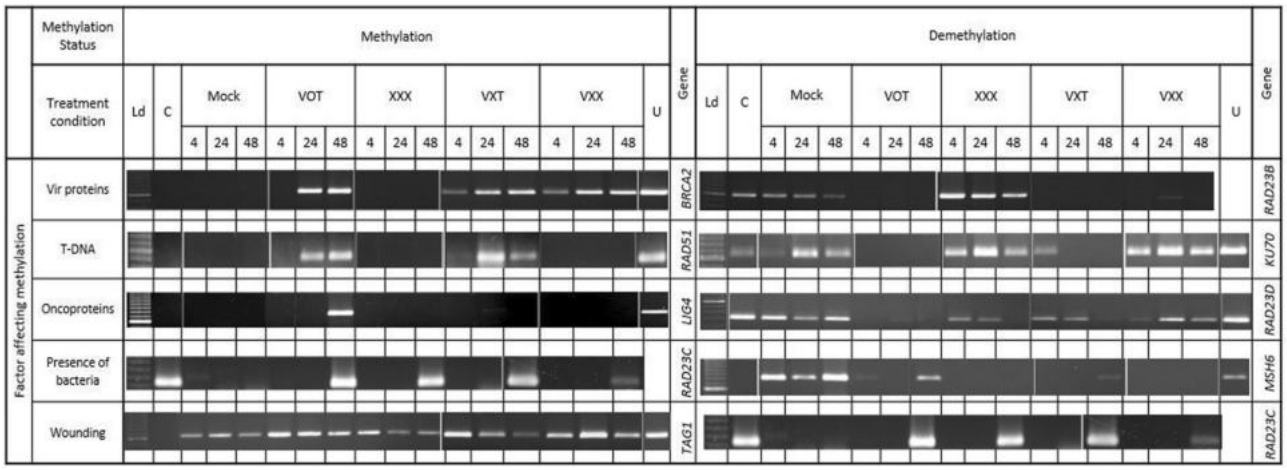

Fig. 3 Gel images of methylation-sensitive restriction digestion PCR showing different methylation patterns exhibited by DNA repair gene promoters in response to various infection conditions, presence of bacteria, oncoproteins, wounding, Vir proteins, and T-DNA. Ld, C, and U are 100 bp ladder, DNA from uninfected control plant, and undigested DNA, respectively. The numbers 4, 24, and 48 indicate three different time intervals in hours. Note that only representative gel images have been shown in this figure and additional images are given in Fig. S8

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), were induced by strains with *Vir* genes. Hence, we inferred that Vir proteins could be the reason for the changes. Similarly, all the promoters were analysed 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 categorize 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 4 h. 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). The expression of *KU70* was upregulated, and its promoter was demethylated due to the influence of T-DNA. Mere presence of bacteria (PAMP) leads 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 leads to expression change did not lead to change in methylation status and vice versa (Table 3). In two other genes *TAG1* and *PMS2L3*, though they 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 to 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.

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	Mock-infection
																		Methylation	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-infection
	P3MAG1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	XRCC1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
HR	*RAD51*	-	-	-	-	-	✓	✓	-	-	-	-	✓	✓	-	-	-	Methylation	T-DNA
	BRCA2	-	-	-	-	-	✓	✓	-	-	-	✓	✓	✓	✓	✓	✓	Methylation	Vir proteins
	ATM	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	BLM	-	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	-	Methylation	Mock-infection
	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 - denote presence and absence of methylation, respectively

*The methylation/demethylation status is only for a short stretch of sequence in the promoter and not the whole promoter

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-infection
				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-infection
	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-infection
	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

*The methylation/demethylation status is only for a short stretch of sequence in the promoter and not the whole promoter

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 (i.e. 17 plants × 3 sets). All the three independent sets (each comprising of DNA from 17 plants) gave the same result, confirming the consistency of 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 × 20 magnification, those with comet patterns appeared to be smaller than the normal ones

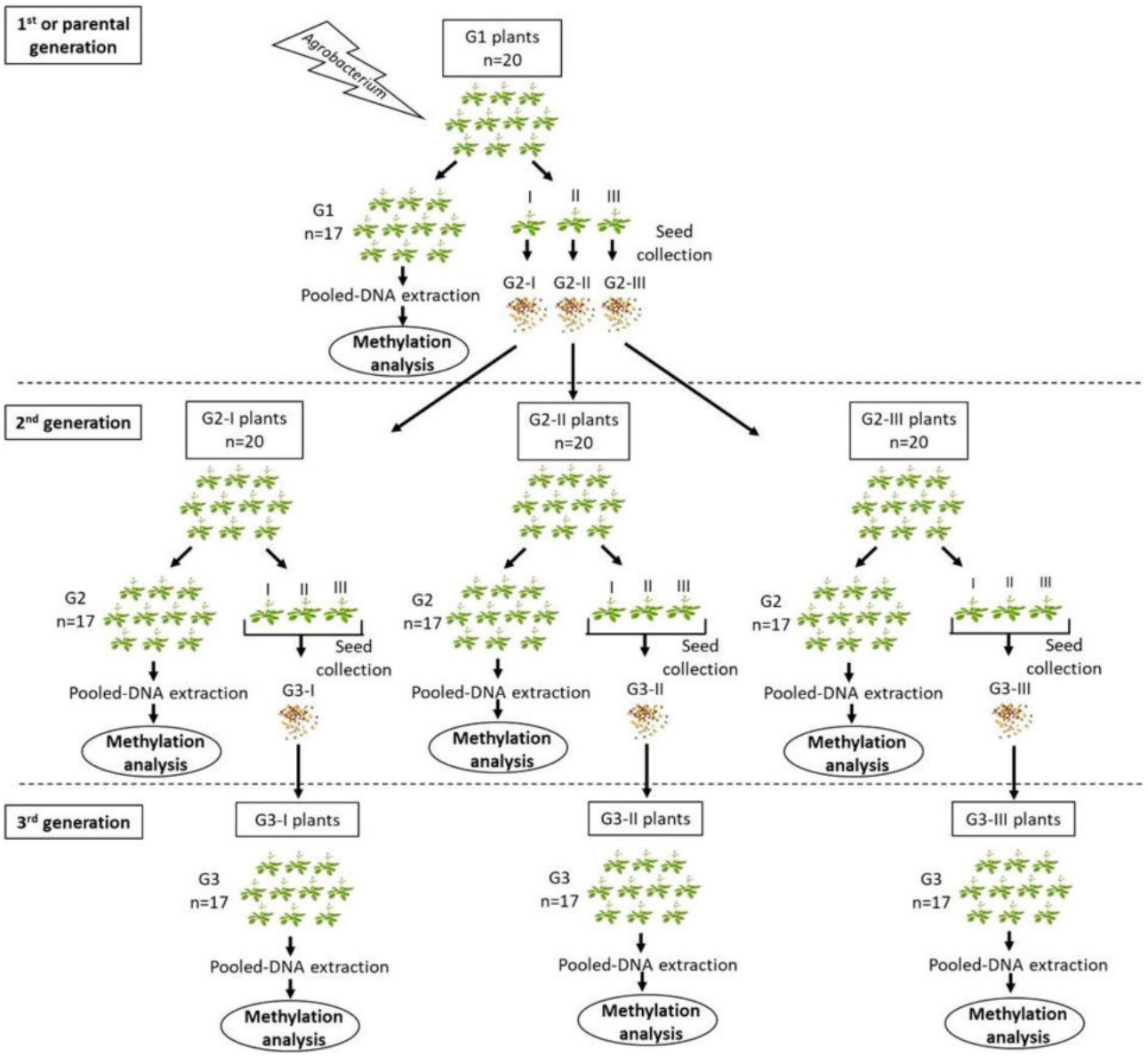

Fig. 4 A detailed outline of the epigenetic study per treatment. G1, G2, and G3 indicate 3 successive generations. 'n' is the number of plants taken in each step

(Fig. 5). 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 bacterium 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). 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

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 infection
		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 infection
		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	-	-	✓	-	-	-		

✓ and - denote presence and absence of methylation, respectively

NER nucleotide excision repair, *BER* base excision repair, *MMR* mismatch repair, *HR* homologous recombination, *NHEJ* non-homologous end joining, *NA* not applicable

*The methylation/demethylation status is only for a short stretch of sequence in the promoter and not the whole promoter

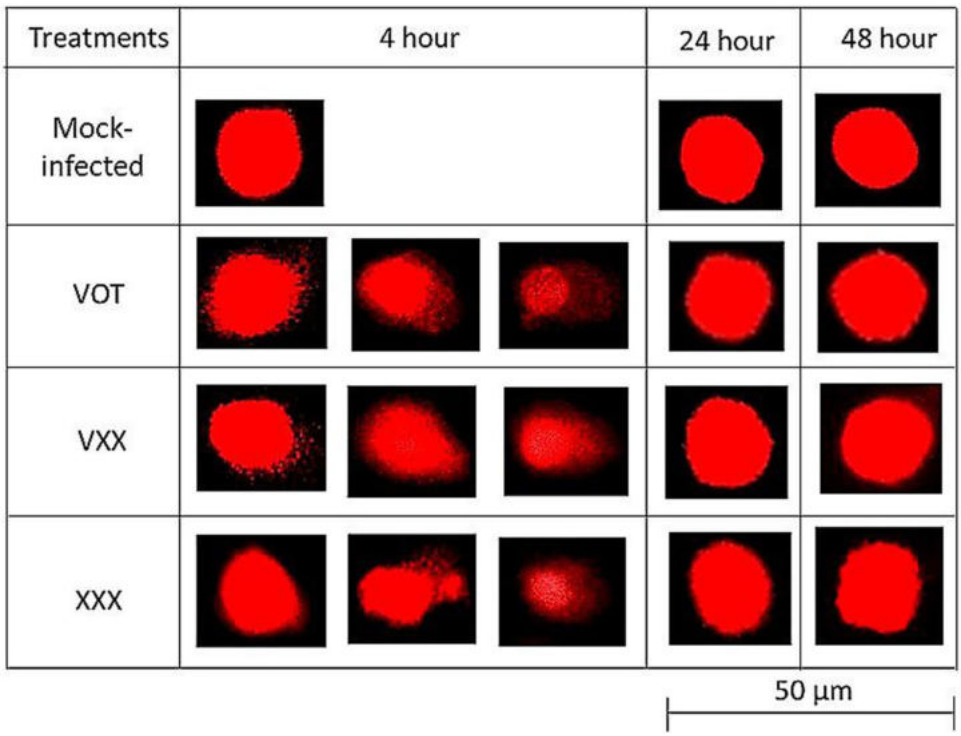

Fig. 5 Host DNA strand break induced by various *Agrobacterium* strains. Three different comet patterns obtained after 4 h of infection and no visible damage at 24 and 48 h post-infection, at × 40 magnification

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) focussed 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 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 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* (*Pst*DC3000) 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 the 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 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 the absence of an NER gene (*XPF*) in *XPF*-deficient *Arabidopsis* mutants led to a small decrease in transformation (Mestiri et al. 2014), the expression of *XPF* increased with respect to mock-infected control 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 *Arabidopsis* 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 orontii* (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 analysed 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 (Table 3). All the remaining genes analysed did not seem to exhibit any correlation between expression and promoter methylation. This was because we have analysed only a small stretch of DNA and not the entire promoter, as a result of which we might have missed other methylated regions. Besides, the MSREs that we used could detect only CpG methylation and not CHG and CHH methylation, where H is any base except G. Also, some other regulatory mechanism involving histone modifications, miRNA or transcription factors, could be involved, which needs further investigation. With the initiative obtained from this study, whole epigenome analysis could be performed that would reveal overall methylation status of promoters and other regulatory regions. It is to be noted that though some of the epigenetic signatures remained in the plant for multiple generations (Fig. S10), 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 leads to hypermethylation of the progeny genome (Boyko et al. 2007; Kathiria et al. 2010). In contrast, reports by Luna et al. (2012) indicate that *Pst*DC3000 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 abovementioned 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 *Pst*DC3000 exhibited resistance against *Pst*DC3000 and *Hyaloperonospora arabidopsidis* and was 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 leads 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 have 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. 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 focussed 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. The 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 with respect to mock-infected control 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 at 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, indicate that T-DNA integration may take place in multiple parallel ways involving random breaking of host DNA or microhomology or any other unknown method. Thoroughly 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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References

Anderson A, Moore L (1979) Host specificity in the genus *Agrobacterium*. Phytopathol 69:320–323

Ascencio-Ibáñez JT, Sozzani R, Lee T-J, Chu T-M, Wolfinger RD, Cella R, Hanley-Bowdoin L (2008) Global analysis of *Arabidopsis* gene expression uncovers a complex array of changes impacting pathogen response and cell cycle during geminivirus infection. Plant Physiol 148:436–454

Bak´o L, Umeda M, Tiburcio AF, Schell J, Koncz C (2003) The VirD2 pilot protein of *Agrobacterium*-transferred DNA interacts with the TATA box-binding protein and a nuclear protein kinase in plants. Proc Natl Acad Sci USA 100:10108–10113

Bartels A, Han Q, Nair P et al (2018) Dynamic DNA methylation in plant growth and development. Int J Mol Sci 19:2144

Baulcombe DC, Dean C (2014) Epigenetic regulation in plant responses to the environment. Cold Spring Harbor Perspect Biol 6:a019471

Boyko A, Kathiria P, Zemp FJ et al (2007) Transgenerational changes in the genome stability and methylation in pathogen-infected plants: (virus-induced plant genome instability). Nucleic Acids Res 35:1714–1725

Cerovska N, Plchova H, Vaculik P, Moravec T, Gichner T (2014) *Potato virus X* induces DNA damage in leaf nuclei of the host plant. *Nicotiana tabacum* L. *var. xanthi*. Biol Plant 58:783–787

de la Riva GA, González-Cabrera J, Vázquez-Padrón R, Ayra-Pardo C (1998) *Agrobacterium tumefaciens*: a natural tool for plant transformation. Electron J Biotechnol 1:118–133

Deeken R, Engelmann JC, Efetova M et al (2006) An integrated view of gene expression and solute profiles of *Arabidopsis* tumors: a genome-wide approach. Plant Cell 18:3617–3634

Deleris A, Halter T, Navarro L (2016) DNA methylation and demethylation in plant immunity. Annu Rev Phytopathol 54:579–603

Dikilitas M, Kocyigit A, Yigit F (2009) A molecular-based fast method to determine the extent of DNA damages in higher plants and fungi. Afr J Biotechnol 8:3118–3127

Ding H, Gao J, Qin C, Ma H, Huang H, Song P, Luo X, Lin H, Shen Y, Pan G, Zhang Z (2014) The dynamics of DNA methylation in maize roots under Pb stress. Int J Mol Sci 15:23537–23554

Ditt F, Kerr KF, de Figueiredo P et al (2006) The *Arabidopsis thaliana* transcriptome in response to *Agrobacterium tumefaciens*. Mol Plant Microbe Interact 19:665–681

Ditt RF, Nester E, Comai L (2005) The plant cell defense and *Agrobacterium tumefaciens*. FEMS Microbiol Lett 247:207–213

Dowen RH, Pelizzola M, Schmitz RJ, Lister R, Dowen JM, Nery JR, Dixon JE, Ecker JR (2012) Widespread dynamic DNA methylation in response to biotic stress. Proc Natl Acad Sci USA E2183–E2191

Durrant WE, Wang S, Dong X (2007) *Arabidopsis* SNI1 and RAD51D regulate both gene transcription and DNA recombination during the defense response. Proc Natl Acad Sci USA 104:4223–4227

Finnegan EJ, Genger RK, Peacock WJ, Dennis ES (1998) DNA methylation in plants. Annu Rev Plant Physiol Plant Mol Biol 49:223–247

Friesner J, Britt AB (2003) Ku80- and DNA ligase IV-deficient plants are sensitive to ionizing radiation and defective in T-DNA integration. Plant J 34:427–440

Gelvin SB (2010) Plant proteins involved in *Agrobacterium*-mediated genetic transformation. Annu Rev Phytopathol 48:45–68

Gelvin SB (2017) Integration of *Agrobacterium* T-DNA into the plant genome. Annu Rev Genet 51:195–217

Gelvin SB, Karcher SJ, DiRita VJ (1983) Methylation of T-DNA in *Agrobacterium tumefaciens* and in several crown gall tumors. Nucleic Acids Res 11:159–174

Gimenez E, Manzano-Agugliaro F (2017) DNA damage repair system in plants: a worldwide research update. Genes 8:299–316

Gohlke J, Deeken R (2014) Plant responses to *Agrobacterium tumefaciens* and crown gall development. Front Plant Sci 5:155–166

Gohlke J, Scholz C-J, Kneitz S et al (2013) DNA methylation mediated control of gene expression is critical for development of crown gall tumors. PLoS Genet 9:e1003267

Heard E, Martienssen RA (2014) Transgenerational epigenetic inheritance: myths and mechanisms. Cell 157:95–109

Heil M, Ton J (2008) Long-distance signalling in plant defence. Trends Plant Sci 13:264–272

Hepburn AG, Clarke LE, Pearson L, White J (1983) The role of cytosine methylation in the control of nopaline synthase gene expression in a plant tumor. J Mol Appl Genet 2:315–329

Hewezi T, Lane T, Piya S, Rambani A, Rice JH, Staton M (2017) Cyst nematode parasitism induces dynamic changes in the root epigenome. Plant Physiol 174:405–420

Hewezi T, Pantalone V, Bennett M, Stewart N, Burch-Smith TM (2018) Phytopathogen-induced changes to plant methylomes. Plant Cell Rep 37:17–23

Iwasaki M, Paszkowski J (2014) Epigenetic memory in plants. EMBO J 33:1987–1998

Jefferson RA (1989) The *GUS*, reporter gene system. Nature 342:837–838

Jiang J, Wang Y, Xie T, Shi X, Wang YP, Sokolov V (2016) DNA methylation analysis during the optimization of *Agrobacterium* mediated transformation of Soybean. Russ J Genet 52:56–63

Joseph JT, Poolakkalody NJ, Shah JM (2019) Screening internal controls for expression analyses involving numerous treatments by combining statistical methods with reference gene selection tools. Physiol Mol Biol Plants 25:289–301

Jupe F, Rivkin AC, Michael TP, Zander M, Motley ST, Sandoval JP, Slotkin RK, Chen H, Castanon R, Nery JR, Ecker JR (2019) The complex architecture and epigenomic impact of plant T-DNA insertions. PLoS genet 15:e1007819

Kathiria P, Sidler C, Golubov A, Kalischuk M, Kawchuk LM, Kovalchuk I (2010) *Tobacco mosaic virus* infection results in an increase in recombination frequency and resistance to viral, bacterial, and fungal pathogens in the progeny of infected tobacco plants. Plant Physiol 4:1859–1870

Kilby NJ, Leyser HM, Furner IJ (1992) Promoter methylation and progressive transgene inactivation in *Arabidopsis*. Plant Mol Biol 20:103–112

Kovalchuk I, Kovalchuk O, Kalck V, Boyko V, Filkowski J, Heinlein M, Hohn B (2003) Pathogen-induced systemic plant signal triggers DNA rearrangements. Nature 423:760–762

Kuśnierczyk A, Winge PER, Jørstad TS, Troczyńska J, Rossiter JT, Bones AM (2008) Towards global understanding of plant defence against aphids–timing and dynamics of early *Arabidopsis* defence responses to cabbage aphid (*Brevicoryne brassicae*) attack. Plant Cell Environ 31:1097–1115

Lacroix B, Citovsky V (2019) Pathways of DNA transfer to plants from *Agrobacterium tumefaciens* and related bacterial species. Annu Rev Phytopathol 57:231–251

Lämke J, Bäurle I (2017) Epigenetic and chromatin-based mechanisms in environmental stress adaptation and stress memory in plants. Genome Biol 18:124–135

Le T-N, Schumann U, Smith NA, Tiwari S, Au PCK, Zhu Q-H, Taylor JM, Kazan K, Llewellyn DJ, Zhang R, Dennis ES, Wang M-B (2014) DNA demethylases target promoter transposable elements to positively regulate stress responsive genes in *Arabidopsis*. Genome Biol 15:458–476

Lee CW, Efetova M, Engelmann JC et al (2009) *Agrobacterium tumefaciens* promotes tumor induction by modulating pathogen defense in *Arabidopsis thaliana*. Plant Cell 21:2948–2962

Lewandowska-Gnatowska E, Kowalczyk LP, Szczegielniak J, Barciszewska M, Barciszewski J, Muszyńska G (2014) Is DNA methylation modulated by wounding-induced oxidative burst in maize? Plant Physiol Biochem 82:202–208

Li JX, Vaidya M, White C, Vainstein A, Citovsky V, Tzfira T (2005) Involvement of KU80 in T-DNA integration in plant cells. Proc Natl Acad Sci USA 102:19231–19236

Linne F, Heidmann I, Saedler H, Meyer P (1990) Epigenetic changes in the expression of the maize A1 gene in *Petunia hybrida*: role of numbers of integrated gene copies and state of methylation. Mol Gen Genet 222:329–336

Löbrich M, Shibata A, Beucher A, Fisher A, Ensminger M, Goodarzi AA et al (2010) γH2AX foci analysis for monitoring DNA double-strand break repair: strengths, limitations and optimization. Cell Cycle 9:662–669

Lucht JM, Mauch-Mani B, Steiner HY, Metraux JP, Ryal J, Hohn B (2002) Pathogen stress increases somatic recombination frequency in *Arabidopsis*. Nat Genet 30:311–314

Luna E, Bruce TJ, Roberts MR, Flors V, Ton J (2012) Next-generation systemic acquired resistance. Plant Physiol 158:844–853

Mathieu O, Reinders J, Caikovski M, Smathajitt C, Paszkowski J (2007) Transgenerational stability of the *Arabidopsis* epigenome is coordinated by CG methylation. Cell 130:851–862

Menke M, Chena I-P, Angelis KJ, Schubert I (2001) DNA damage and repair in *Arabidopsis thaliana* as measured by the comet assay after treatment with different classes of genotoxins. Mutation Res 493:87–93

Mestiri I, Norre F, Gallego ME, White CI (2014) Multiple host-cell recombination pathways act in *Agrobacterium*-mediated transformation of plant cells. Plant J 77:511–520

Nougayrède JP, Homburg S, Taieb F, Boury M, Brzuszkiewicz E, Gottschalk G, Buchrieser C, Hacker J, Dobrindt U, Oswald E (2006) *Escherichia coli* induces DNA double-strand breaks in eukaryotic cells. Science 313:848–851

Park SY, Vaghchhipawala Z, Vasudevan B et al (2015) *Agrobacterium* T-DNA integration into the plant genome can occur without the activity of key non-homologous end-joining proteins. Plant J 81:934–946

Pavet V, Quintero C, Cecchini NM, Rosa AL, Alvarez ME (2006) *Arabidopsis* displays centromeric DNA hypomethylation and cytological alterations of heterochromatin upon attack by *Pseudomonas syringae*. Mol Plant Microbe Interact 19:577–587

Philips GJ, Dudley KJ, Waterhouse PM, Hellens RP (2019) The rapid methylation of T-DNAs upon *Agrobacterium* inoculation in plant leaves. Front Plant Sci 10:312–329

Riballo E, Kuhne M, Rief N, Doherty A, Smith GC, Recio MJ et al (2004) A pathway of double-strand break rejoining dependent upon ATM, Artemis and proteins locating to gamma-H2AX foci. Mol Cell 16:715–724

Rogers SO, Bendich AJ (1988) Extraction of total cellular DNA from plants. In: Gelvin SB, Schilperoort RA (eds) Plant molecular biology manual. Kluwer Academic, Netherlands, pp 183–190

Sánchez AL, Stassen JH, Furci L, Smith LM, Ton J (2016) The role of DNA (de)methylation in immune responsiveness of *Arabidopsis*. Plant J 88:361–374

Sardesai N, Subramanyam S (2018) *Agrobacterium*: a genome-editing tool-delivery system. *Agrobacterium* Biology. Springer, Cham, pp 463–488

Satgé C, Moreau S, Sallet E, Lefort G, Auriac M-C, Remblière C, Cottret L, Gallardo K, Noirot C, Jardinaud M-F, Gamas P (2016) Reprogramming of DNA methylation is critical for nodule development in *Medicago truncatula*. Nature Plants 2:16166

Shah JM, Ramakrishnan AM, Singh AK et al (2015) Suppression of different classes of somatic mutations in *Arabidopsis* by *vir* gene-expressing *Agrobacterium* strains. BMC Plant Biol 15:210–223

Song J, Bent AF (2014) Microbial pathogens trigger host DNA double-strand breaks whose abundance is reduced by plant defense responses. PLoS Pathog 10:e1004030

Song J, Durrant WE, Wang S, Yan S, Tan EH, Dong X (2011) DNA repair proteins are directly involved in regulation of gene expression during plant immune response. Cell Host Microbe 9:115–124

Tenea GN, Spantzel J, Lee LY, Zhu Y, Lin K, Johnson SJ, Gelvin SB (2009) Overexpression of several *Arabidopsis* histone genes increases *Agrobacterium*-mediated transformation and transgene expression in plants. Plant Cell 21:3350–3367

Tomlinson AD, Fuqua C (2009) Mechanisms and regulation of polar surface attachment in *Agrobacterium tumefaciens*. Curr Opin Microbiol 12:708–714

Tukey JW (1953) Some selected quick and easy methods of statistical analysis. Trans NY Acad Sci 16:88–97

Vaghchhipawala ZE, Vasudevan B, Lee S, Morsy MR, Mysore KS (2012) *Agrobacterium* may delay plant nonhomologous end-joining DNA repair via XRCC4 to favor T-DNA integration. Plant Cell 24:4110–4123

van Attikum H, Bundock P, Hooykaas PJJ (2001) Non-homologous end-joining proteins are required for *Agrobacterium* T-DNA integration. EMBO J 20:6550–6558

van Attikum H, Hooykaas PJJ (2003) Genetic requirements for the targeted integration of *Agrobacterium* T-DNA in *Saccharomyces cerevisiae*. Nucleic Acids Res 31:826–832

van Kregten M, de Pater S, Romeijn R, van Schendel R, Hooykaas PJJ, Tijsterman M (2016) T-DNA integration in plants results from polymerase-theta-mediated DNA repair. Nature Plants 2:16164

Veena JH, Doerge RW, Gelvin SB (2003) Transfer of T-DNA and Vir proteins to plant cells by *Agrobacterium tumefaciens* induces expression of host genes involved in mediating transformation and suppresses host defense gene expression. Plant J 35:219–236

Wada Y, Miyamoto K, Kusano T, Sano H (2004) Association between up-regulation of stress-responsive genes and hypomethylation of genomic DNA in tobacco plants. Mol Genet Genomics 271:658–666

Wang B, Liu J, Tian Z, Song B, Xie C (2005) Monitoring the expression patterns of potato genes associated with quantitative resistance to late blight during *Phytophthora infestans* infection using cDNA microarrays. Plant Sci 169:1155–1167

Wang S, Durrant WE, Song J, Spivey NW, Dong X (2010) *Arabidopsis* BRCA2 and RAD51 proteins are specifically involved in defense gene transcription during plant immune responses. Proc Natl Acad Sci USA 107:22716–22721

Winter D, Vinegar B, Nahal H, Ammar R, Wilson GV, Provart NJ (2007) An "Electronic Fluorescent Pictograph" browser for exploring and analyzing large-scale biological data sets. PLoS ONE 2:e718

Woo HR, Pontes O, Pikaard CS, Richards EJ (2007) VIM1, a methylcytosine-binding protein required for centromeric heterochromatinization. Genes Dev 21:267–277

Yan S, Wang W, Marqués J, Mohan R, Saleh A, Durrant WE, Song J, Dong X (2013) Salicylic acid activates DNA damage responses to potentiate plant immunity. Mol Cell 52:602–610

Zhang H, Lang Z, Zhu JK (2018) Dynamics and function of DNA methylation in plants. Nat Rev Mol Cell Biol 19:489–506