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A stage-dependent seed defense response to explain efficient seed transmission of *Xanthomonas citri* pv. *fuscans* to common bean

Armelle Darrasse | Łukasz Paweł Tarkowski | Martial Briand | David Lalanne | Nicolas W. G. Chen | Matthieu Barret | Jerome Verdier

University Angers, Institut Agro, INRAE, IRHS, SFR QUASAV, Angers, France

Correspondence
Jerome Verdier, University Angers, Institut Agro, INRAE, IRHS, SFR QUASAV, F-49000 Angers, France.
Email: jerome.verdier@inrae.fr

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Abstract

Although seed represents an important means of plant pathogen dispersion, the seed–pathogen dialogue remains largely unexplored. A multiomic approach was performed at different seed developmental stages of common bean (*Phaseolus vulgaris* L.) during asymptomatic colonization by *Xanthomonas citri* pv. *fuscans* (*Xcf*), At the early seed developmental stages, we observed high transcriptional changes both in seeds with bacterial recognition and defense signal transduction genes, and in bacteria with up-regulation of the bacterial type 3 secretion system. This high transcriptional activity of defense genes in *Xcf*-colonized seeds during maturation refutes the widely diffused assumption considering seeds as passive carriers of microbes. At later seed maturation stages, few transcriptome changes indicated a less intense molecular dialogue between the host and the pathogen, but marked by changes in DNA methylation of plant defense genes, in response to *Xcf* colonization. We showed examples of pathogen-specific DNA methylations in colonized seeds acting as plant defense silencing to repress plant immune response during the germination process. Finally, we propose a novel plant–pathogen interaction model, specific to the seed tissues, highlighting the existence of distinct phases during seed–pathogen interaction with seeds being actively interacting with colonizing pathogens, then both belligerents switching to more passive mode at later stages.

KEYWORDS
dialogue, dual transcriptomics, epigenome, *Phaseolus vulgaris*

1 | INTRODUCTION

Legumes provide a sustainable source of proteins for human and livestock diet, moreover their symbiotic nitrogen fixation capacity contributes to soil preservation and reduces the need for chemical fertilizers (Ferreira et al., 2021; Stagnari et al., 2017). An important factor limiting legume utilization is their relatively high yield variability, greatly due to their susceptibility to environmental factors such as biotic and abiotic stresses (Cernay et al., 2015; Martins et al., 2020). While legumes are expected to better perform under

changing climatic conditions in relation to other crops thanks to higher biomass accumulation under increased atmospheric CO_2 levels and higher photosynthetic efficiency under increased irradiation levels, other traits are predicted to be negatively affected, such as seed quality and resistance to pathogens (Myers et al., 2014).

Pathogens are responsible for 35%–70% yield losses on grain legumes (Martins et al., 2020). An important determinant of disease outbreak is pathogen dispersal through infected seeds (Denancé & Grimault, 2022). The mode of transmission of pathogens to the seed can be schematically summarized in three nonexclusive pathways: internal (via the host xylem), floral (via the pistil) and external as a consequence of contact of the seed with symptomatic fruit tissues or with threshing residues (Maude, 1996). For instance, *Xanthomonas citri* pv. *fuscans* (*Xcf*), causal agent of common bacterial blight of bean (CBB), can use these three pathways for its transmission to common bean seeds (Darrasse et al., 2018; Darsonval et al., 2008). Infected seeds can be symptomatic or asymptomatic, and are generally associated with high or moderate bacterial population sizes, respectively, moreover symptomatic seeds often fail to germinate (Chen, Ruh, et al., 2021; Darrasse et al., 2018) and no viable pathogen control method to counteract bacterial seed infections exists.

Decades of research led to a comprehensive overview of the genetic (for review see, Dodds & Rathjen, 2010; Wirthmueller et al., 2013) and epigenetic (for review see, Hannan Parker et al., 2022) mechanisms involved in plant–pathogen interactions during vegetative growth. However, the molecular dialogue that takes place between seeds and pathogens was overlooked to date. On the plant side, in the event of an incompatible interaction between *Medicago truncatula* and *Xanthomonas campestris* pv. *campestris* (*Xcc*), seed transcriptome exhibited an activation of defense response and a repression of seed maturation pathways (Terrasson et al., 2015). From the bacterial side, some specific genetic determinants such as the type 3 secretion system (T3SS, Darsonval et al., 2008) and adhesins (Darsonval et al., 2009) were shown to be involved in the transmission of *Xcf* to common bean seeds. Involvement of the T3SS in seed transmission was also documented for *Acidovorax citrulli* in watermelon (Dutta et al., 2014). However, a global view of bacterial transcriptomic changes occurring during seed transmission is currently missing. This lack of knowledge is partly due to the difficulties of collecting enough bacterial RNA from the seeds. Indeed seed-associated bacterial population sizes are usually very low (from 10 to 1000 colony-forming unit [CFU] per bean seed; Chesneau et al., 2022) and follow a Poisson distribution, which complicates the sampling of infected seeds and prevent molecular analysis of seed–pathogens interactions (Gitaitis & Walcott, 2007).

Since knowledge regarding molecular interactions occurring during bacterial seed infections is currently lacking, the objective of this work was to decipher the molecular dialogue between the common bean (*Phaseolus vulgaris* L.) seed and a seed pathogen at several stages of seed development to identify major molecular factors involved in seed infection establishment and pathogen transmission to the seedling. A dual RNA-sequencing (RNA-seq) approach to identify both the host seed and the *Xcf* pathogen transcriptomes was performed at three stages of seed development during seed filling, seed maturation and seed maturity. The technical limitation of low bacterial population within seeds was successfully bypassed using bacterial transcript enrichment. This transcriptomic analysis was complemented by the analysis of small RNAs (sRNAs) and DNA methylation changes in infected seeds to reveal the role of these mechanisms in the seed–pathogen interaction, which allowed us to propose a novel model in plant-pathogen interactions specific to seed developmental stage and explaining the efficiency of pathogen seed transmission.

2 | MATERIALS AND METHODS

2.1 | Bacterial strain and inoculum preparation

The *Xcf* bacterial strain 7767R (Rif^R, Darrasse et al., 2018) was grown for 24 h at 28°C in Tryptic Soy Agar at 10% (1.7 g L^{-1} tryptone, 0.3 g L^{-1} soybean peptone, 0.25 g L^{-1} glucose, 0.5 g L^{-1} NaCl, 0.5 g L^{-1} K_2HPO_4 and 15 g L^{-1} agar) supplemented with 50 mg L^{-1} rifamycin. Bacterial cells were suspended in sterile distilled water, calibrated at 10^8 CFU mL^{-1} (OD_{600} = 0.1) and adjusted to 10^6 CFU mL^{-1} for spray-inoculation.

2.2 | Plant materials and production of infected seeds

Experiments were performed with *Phaseolus vulgaris* L. cv. Flavert, a cultivar susceptible to CBB (Darrasse et al., 2007). To produce plants for flower inoculation, seeds were sown in one liter of Tray substrate (NF U 44–551, Klasmann-Deilmann GmbL). Plants were grown in a controlled growth chamber with 16 h of light at 23°C and 8 h of dark at 20°C and a relative humidity (RH) of 70%. Plants were watered twice a week during the first 3 weeks, then with a nutrient solution (N/P/K = 15/10/30). Plants were staked and pinched after the third leaf.

Plants were spray-inoculated at the flower bud stage (R5, Michael 1994) with either *Xcf* bacterial suspension (10^6 CFU mL^{-1}) or water as control. The day before inoculation, temperature (day 25°C/night 23°C) and RH (95%) were increased. Inoculation was performed using a two-step protocol. First, small green flower buds were sprayed. Three days later, flower buds at the pollination stage were tagged. Then, a second inoculation was performed at 1 day after pollination (DAP) when tagged organs turned into open flowers. Then afterward, RH was reduced to 70% to limit pathogen symptom development and seed abortion. Three independent replicates of five plants (n = 15) were inoculated. For each replicate of five plants, seeds were collected from tagged pods at 24, 35 and 42 DAP, aseptically to avoid contamination by external bacterial populations (Darsonval et al., 2008), and bulked into seed lots.

2.3 | Monitoring of bacterial population sizes

For each sample, *Xcf* population sizes were determined from 10 seeds and from 5 pools of 3 seeds. Seeds were soaked in 0.5 mL of sterile water per seed overnight at 4°C under shaking (150 rpm). Then, 50 µL of serial dilutions were plated on 10% TSA. Colonies were monitored 5 days after incubation at 28°C. The infection rate of a sample (p) was calculated from the analysis of N subsamples according to the formula $p = 1-(Y/N)^{1/n}$ (Maury et al., 1985), where n is the number of seeds in each group and Y the number of mock-treated groups.

2.4 | Seed physiological analyses

Three subsamples of 10 seeds were used to determine dry weight and water content. Each subsample was weighed before and after incubation (3 days) in a 96°C incubator (Memmert).

2.5 | Plant and bacterial RNA extraction and RNA-seq

Seed samples harvested at 24, 35 and 42 DAP were flash-frozen in liquid nitrogen. Samples were ground in liquid nitrogen using a mechanical grinder (Retsch MM300 TissueLyser) during 1 min at 30 Hertz. Total RNAs were extracted using the NucleoSpin® RNA Plant and Fungi Kit (Macherey-Nagel), according to the manufacturer instructions. RNA quantity and integrity were assessed respectively using a NanoDrop ND-1000 (NanoDrop Technologies) and a 2100 Bioanalyzer (Agilent Technologies). Library constructions and single-end sequencing (SE50, 20 M) were outsourced to the Beijing Genomics Institute (BGI, https://www.bgi.com) using the Illumina Hiseq 2500 technology. Raw reads are available at GSE226918.

Using the same *Xcf*-inoculated seed lot as for plant RNAs, pellets enriched in bacteria were collected after soaking *Xcf-colonized* seeds (2 mL per gram of seed) overnight in KPO_4 buffer, (50 mM, pH 6.8), supplemented with 20% of blocking agent (RNAlater; Thermo Fisher Scientific). After centrifugation (15 min at 15 000*g*) and removal of the supernatant, total RNAs were extracted from the pellets enriched in bacteria as previously described (Darsonval et al., 2009). Concentration and integrity of RNAs were assessed with Qubit (Invitrogen) and a 2100 Bioanalyzer (Agilent Technologies), respectively. As total RNA extracted from pellet enriched in bacteria corresponded mainly to plant transcripts (not shown), we designed a procedure of bacterial transcript enrichment. Bacterial messenger RNAs (mRNAs) were captured using the SureSelectXT RNA Direct technology (Agilent). A total of 54 548 probes of 120-nts length were designed based on the predicted mRNAs of *Xcf*7767R genome sequence (GCA_900234465; Chen et al., 2018). Quality and quantity of sequencing libraries were evaluated and quantified using Bioanalyzer and KAPA Library Quantification assay (Roche). Paired-end sequencing (2 × 75 bp) was performed with a NextSeq 550 System High OutPut Kit (Illumina). Raw reads are available at GSE227386.

After quality control, high-quality reads were mapped either on *Xcf* 7767R transcriptome (Briand et al., 2021) (https://bbric-pipelines.toulouse.inra.fr/myGenomeBrowser?browse=1&portalname=Xcf7767Rpb&owner=armelle.darrasse@inrae.fr&key=TwzQ08DA) or on *P. vulgaris* transcriptome version 2.1 (https://phytozome-next.jgi.doe.gov/info/Pvulgaris_v2_1) using quasi-mapping alignment and quantification methods of Salmon algorithm v.1.2 (Patro et al., 2017). RNA-Seq data were normalized as transcripts per million. Differentially expressed genes (DEGs) were determined using DESeq2 v1.22.2 (Love et al., 2014), using an adjusted $p < 5\%$ following a Benjamini–Hochberg procedure. *Xcf* DEGs were analyzed between sampling dates. *P. vulgaris* DEGs were obtained by comparing *Xcf*- versus H_2O-inoculated seeds at each developmental stage. Gene annotations were provided with the *P. vulgaris* version 2.1 genome and Mapman functional categories v.4 were determined using Mercator tool from the predicted protein sequences (Schwacke et al., 2019). Bacterial type III effectors were predicted using the automated machine-learning based web server Effectidor (Wagner et al., 2022). Over representation analyses of MapMan or clusters of orthologous groups (COGs) terms were performed, respectively for plant and bacteria DEGs, using clusterProfiler (Yu et al., 2012) package in R by applying an adjusted p-value cut-off < 0.05 obtained after the Bonferroni-Hochberg procedure.

DEGs during seed germination were identified using the data generated by Narsai et al. (2017) available in the Sequence Read Archive database (accession GSE94457). Raw reads were downloaded and mapped against the Arabidopsis transcriptome using Salmon algorithm and DEGs during germination kinetic were determined using ImpulseDE2 algorithm (Fischer et al., 2018) following an adjusted $p < 1\%$.

2.6 | qRT-PCR experiments at postgermination stage

To determine genes involved in postgermination defense, we inoculated mock-treated seeds with 10^7 of *Xcf* CFU mL^{-1} (called Mock–Xcf), *Xcf*-colonized seeds with 10^7 of *Xcf* CFU mL^{-1} (called Xcf–Xcf) and mock-treated seeds with H_2O (called Mock–Mock) during 25 min followed by 3 min of vacuum infiltration before keeping them at room temperature for 3 h, corresponding to a inoculation time during postgermination of about 3 h. RNAs were extracted using the NucleoSpin® RNA Plant and Fungi Kit (Macherey-Nagel) as described above but including a DNAse treatment (Macherey-Nagel; rDNAse set). RNA were quantified using a using a NanoDrop ND-1000 (NanoDrop Technologies) and cDNA was synthesized from 1 µg of total RNA using the Reverse Transcription system (iScriptTM cDNA Synthesis Kit; Bio-Rad). Quantitative Real time PCR was performed using Sybr Green Master Mix (SYBR Green master mix; Bio-Rad) on a CFX96 real-time detection system (Bio-Rad Laboratories). *EF1* and *UBI* genes were used as housekeeping

genes as described in Darrasse et al. (2010). Expression values were normalized using the $\Delta\Delta C_t$ method by normalizing first against the geometric mean of housekeeping genes then against the mock–mock treatment. To visualize changes, we Log_2-transformed the expression values. Primers used for real-time PCR are listed in Table S4.

2.7 | sRNA sequencing extraction and analysis

Using the same frozen powders used for RNA-seq experiments and obtained from *Xcf*- and H_2O-inoculated seeds from 24 DAP and 42 DAP, we extracted sRNA using the NucleoSpin® miRNA Kit (Macherey-Nagel), according to the manufacturer's instructions. sRNA enrichment was validated using Bioanalyzer small RNA analysis. sRNAs were sequenced using DNBseq sequencing technology (SE50 40M, BGI) and Unique Sequence identifiers to correctly quantify unique reads. Reads of 20 to 24 nucleotides were extracted and mapped on the reference mature microRNA (miRNA) database available in miRBase version 22 (Kozomara et al., 2019) using bowtie (Langmead et al., 2009) and quantified using SAMtools (Li et al., 2009). Differentially expressed sRNA between *Xcf*-inoculated versus H_2O-inoculated seeds at 24 and 42 DAP were determined using DESeq. 2 following a *p*-value threshold < 5% from the SARTools R package (Varet et al., 2016). Known and putative novel sRNAs were mapped to the *P. vulgaris* genome sequence using ShortStack4 algorithm (Johnson et al., 2016) and displayed in the dedicated Jbrowse https://iris.angers.inrae.fr/pvulgaris_v2 in the 'small RNA tracks' section. Transcripts potentially targeted by miRNAs were predicted via analyzing complementary matching between sRNA and target and evaluating target site accessibility using psRNATarget tool (Dai & Zhao, 2011; Dai et al., 2018) and a threshold of expectation below 5 was set to consider transcripts as putative miRNA targets. Raw reads are publicly available at GSE226920.

2.8 | Plant DNA extraction and bisulfite sequencing experiments

From the same frozen seed powders used for mRNA and sRNA extractions, we performed DNA extraction, on the three biological replicates of *Xcf*- and H_2O-inoculated seeds at 42 DAP, using the NucleoSpin® DNA Food Kit (Macherey-Nagel), according to the manufacturer's instructions. DNA samples were sent to the BGI Genomics for bisulfite treatment using a ZYMO EZ DNA Methylation-Gold Kit, library construction and paired-end sequencing using BGISEQ-500 sequencing technology (PE100 45M). FastQC was used to check sequencing quality and clean reads were mapped to the *P. vulgaris* genome version 2.1 using Bismark software (Krueger & Andrews, 2011). After mapping, deduplication of sequences and quantification of cytosine methylation were performed using Bismark_deduplicate and Bismark_methylation_extractor. Each context of methylation was considered independently: CG, CHG or CHH and corresponding bigwig files were generated using bismark_to_bigwig python script and displayed in the dedicated Jbrowse: https://iris.angers.inrae.fr/pvulgaris_v2. Putative differentially methylated regions (DMRs) were identified in each independent methylation context using DMRCaller algorithm available in R (Catoni et al., 2018). Raw reads are publicly available at https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE226919.

3 | RESULTS

3.1 | Seed transmission of moderate *Xcf* population sizes does not impact seed development

Seed transmission of *Xcf* 7767R was investigated following spray-inoculation of *P. vulgaris* L. cv Flavert. Three stages of seed development were targeted: (i) 24 DAP (seed filling), (ii) 35 DAP (seed maturation) and 42 DAP (seed maturity). Seed water content (Figure 1a) and dry seed weight (Figure 1b) were not significantly impacted by *Xcf* inoculation. As described in Darsonval et al. (2008), we used 10^6 CFU mL^{-1} for *Xcf* spray inoculation at flowering time to allow seed bacterial transmission without the apparition of symptoms during seed development. Otherwise, higher concentrations could generate symptomatic seed bacterial transmission leading to defect in the germination of infected seeds. Following this mild treatment, about 80% of seeds were contaminated with *Xcf* with an average population size of 10^5 CFU g^{-1} of seeds at 24 DAP (Figure 1c). Over the course of seed development, the frequency of detection of *Xcf* decreased from 80% to 50%. This was accompanied by a significant decrease in *Xcf* population size from 35 to 42 DAP, down to an average of 10^3 CFU g^{-1} of seeds at maturity (Figure 1c).

3.2 | Changes in the *Xcf* bacterial transcriptome during seed development

To explore the genetic determinants involved in *Xcf* seed transmission, dual (host and pathogen) transcriptome sequencing was performed at 24, 35 and 42 DAP. An essential step to obtain sufficient bacterial transcript data was to enrich RNA-seq libraries for *Xcf* transcripts using 54 656 capture probes. Among a total of 27.7–61.3 M sequenced reads that were obtained for each sample, 4.7–55.1 M mapped on the predicted transcriptome of *Xcf* strain 7767R (Table S1). A total of 4372 mRNAs were detected in at least one sample (count ≥10), which corresponded to >96% of the 4537 predicted mRNAs, thus validating our *Xcf* transcriptome enrichment strategy. In the absence of reference condition, bacterial transcriptomes were compared between the different sampling dates. Extensive changes in *Xcf* transcriptome were observed between seed filling (24 DAP) and the two other seed maturation stages (35 and 42 DAP). Indeed, 865 and 1674 DEGs were detected between 24 and 35 DAP and 24 and 42 DAP, respectively, (Figure 2a). On the other hand, only 17 DEGs were detected between 35 and 42 DAP, indicating that transcriptomic levels stabilized between seed

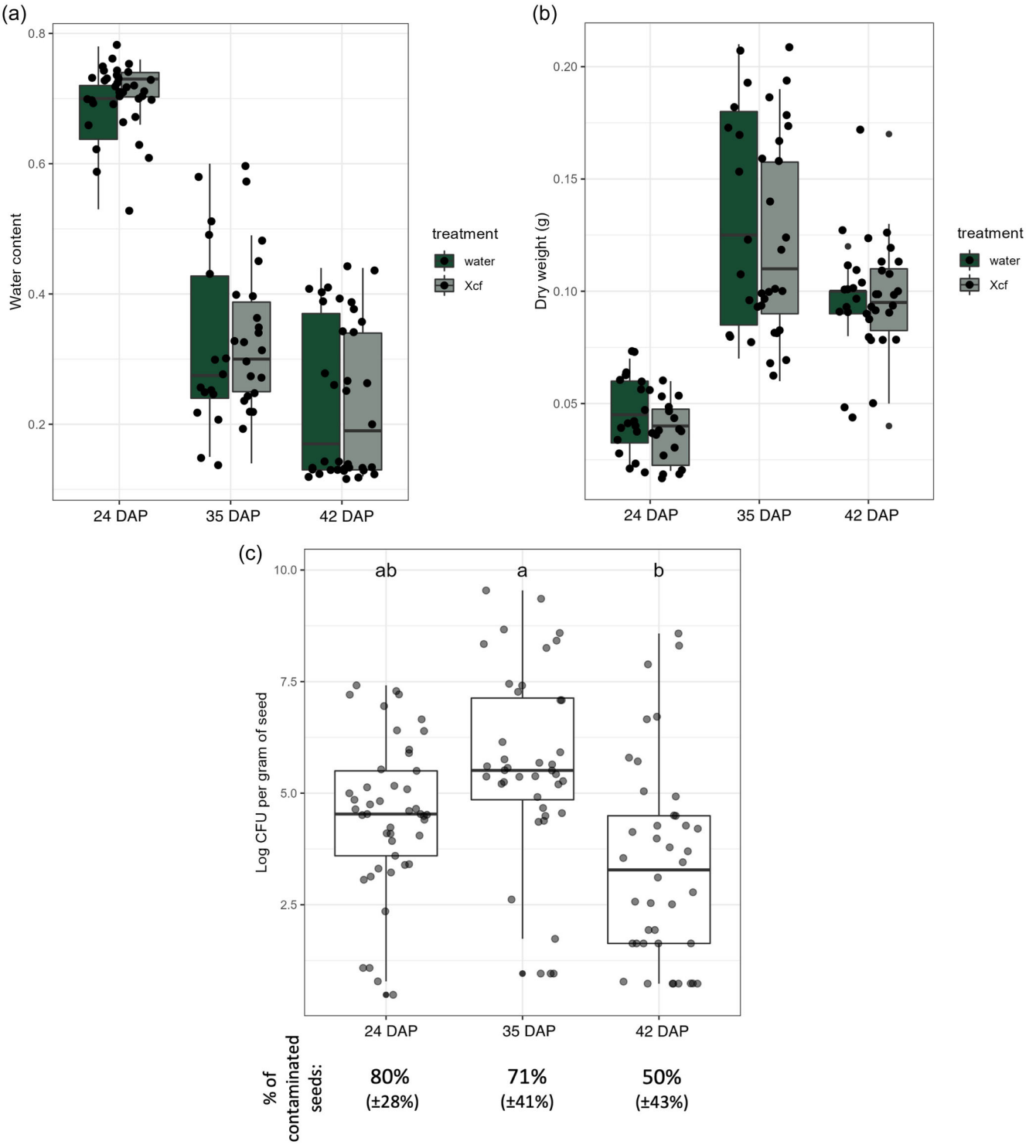

FIGURE 1 Transmission of *Xcf* to bean seeds. (a) Seed water content, (b) seed dry weight (gram) and (c) *Xcf* population size (log_{10} CFU per gram of seed) at the different sampling stages (24 DAP, 35 DAP and 42 DAP). Differences between the sampling stage and the treatment (H_2O- or *Xcf*-inoculated) were assessed by Kruskall–Wallis test followed by post hoc Dunn's test. The percentages of observed contaminated seeds at different seed developmental stages are indicated (expressed as averages with SD between brackets). CFU, colony-forming unit; DAP, day after pollination; *Xcf*, *Xanthomonas citri* pv. *fuscans*. [Color figure can be viewed at wileyonlinelibrary.com]

maturation and maturity stages. In line with this result, over-representation analyses of COG terms associated to bacterial DEGs were performed and revealed that intracellular trafficking and secretion terms were enriched at 24 DAP and posttranslational modification at 35 and 42 DAP (Figure 2b). The other enriched categories were translation and reparation/repair, both enriched at 42 DAP, and extracellular structure and cell motility, both enriched at 24 DAP (Figure 2b).

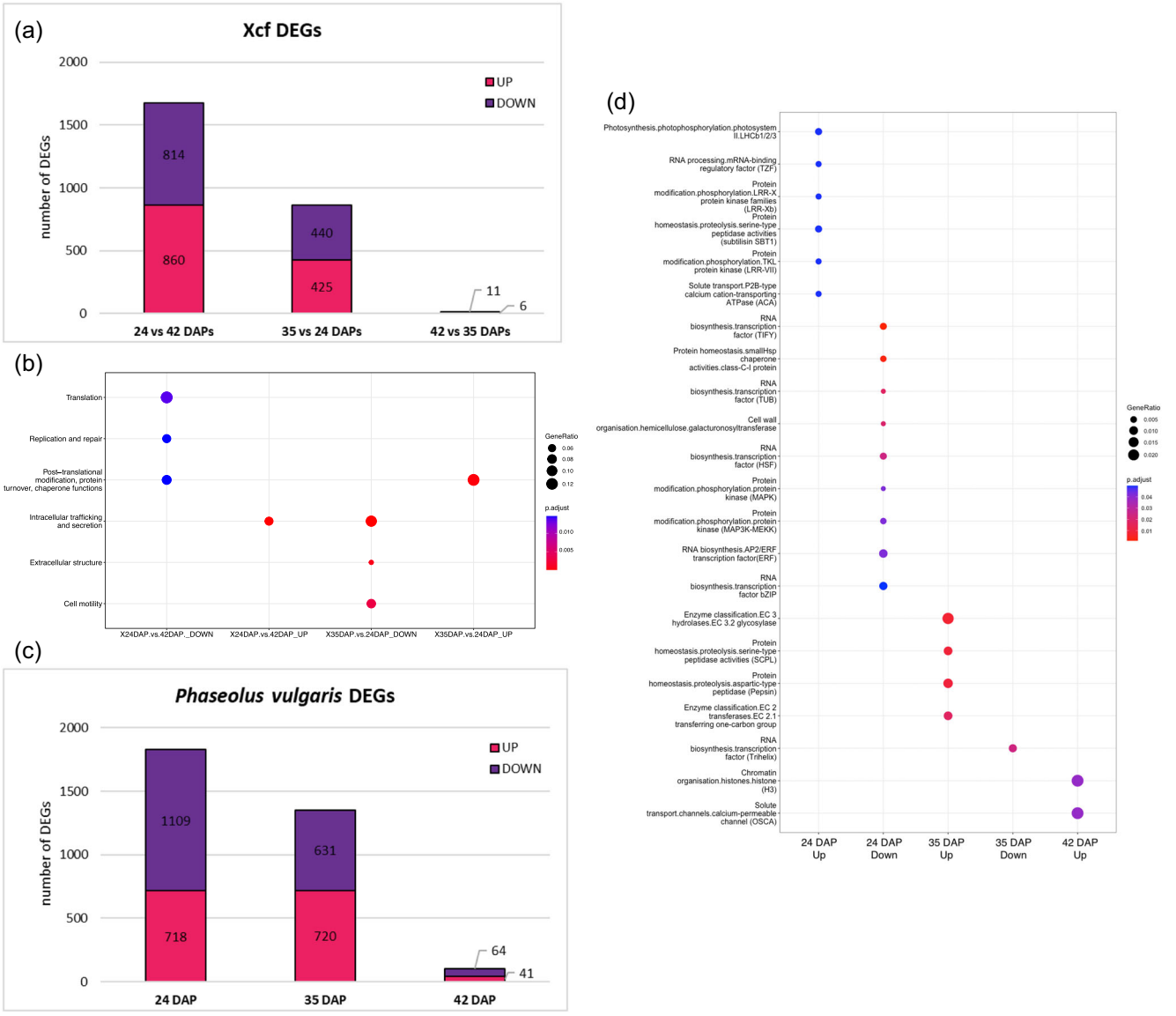

FIGURE 2 Dual transcriptomic analysis of the *Xcf–Phaseolus vulgaris* seed interaction. (a) and (c) Histograms summarizing the number of differentially expressed genes (DEGs) detected comparing datasets from different seed development stages from *Xcf* samples (a) and DEGs from different development stages from *P. vulgaris* samples (c). The number of DEGs is indicated on the bars. (b) and (d) Dot plots showing category enrichment results obtained through gene ontology analysis of DEGs from *Xcf* (b) and *P. vulgaris* (d). Gene ontology analysis was performed with the clusterProfiler package for R. DAP, day after pollination; mRNA, messenger RNA; *Xcf*, *Xanthomonas citri* pv. *fuscans*. [Color figure can be viewed at wileyonlinelibrary.com]

A manual annotation on the COG term related to secretion processes, enriched at 24 DAP, revealed that many T3SS encoding genes and several *xps* genes involved in the T2SS were up-regulated at the seed filling stage, but not later during seed maturation (Table S1). This was consistent with the observed up-regulation of the master regulator *hrpG* that is known to control many genes involved in the interaction with the host plant (Teper et al., 2021) such as the T3SS transcriptional activator *hrpX* and cognate effectors (T3Es) but also the *xps* genes involved in the secretion of cell wall degrading enzymes (Szczesny et al., 2010). In line with this result, 26/40 (65%) of T3E-encoding genes and several genes encoding pectin lyase (1), pectate lyases (2), glycoside hydrolases (34) and proteases (40) were only up-regulated at early stage (Table S1). As demonstrated in Darsonval et al. (2008), T3SS encoding genes, including hrpG and hrpX are essential genes for efficient bacterial seed transmission. Together, these results suggested that bacteria were actively interacting with the host plant only at early seed maturation stages, but not later.

3.3 | Transcriptomic analysis of bean seeds in response to pathogen colonization

Changes in *P. vulgaris* transcriptome were assessed using the same seed lots as for the *Xcf* transcriptome analyses described above. All results from this RNA-seq analysis are displayed in the dedicated

Jbrowse (https://iris.angers.inrae.fr/pvulgaris_v2) and in Table S1. Similar to what was observed in *Xcf* transcriptome changes, RNA-seq analysis between *Xcf*-colonized versus mock-treated seeds revealed that the plant response to the bacteria was higher at early than later stages of seed maturation, with 1826 DEGs at 24 DAP, 1351 at 35 DAP and only 105 at 42 DAP (Figure 2c). Only 137 DEGs (7.5% of 24 DAP DEGs) were shared between 24 and 35 DAP, indicating that the plant's response was different between these stages, ending up with almost no response in mature seeds. Only one DEG, encoding a chaperone protein DnaJ-like protein, was found to be in common between all the three stages (*Phvul.001G262000)* and could reflect a cellular stress in seeds inoculated with *Xcf*. This low overlap in DEGs across different seed developmental stages was also reflected at the level of functional category enrichments, which were different between 24, 35 and 42 DAP (Figure 2d). The 24 DAP timepoint displayed the most complex response, with six up-regulated and nine down-regulated Mapman functional categories detected through functional enrichment analysis of DEGs. Some categories had well characterized roles in the plant-microbe molecular interactions, such as leucine rich repeat protein kinases (LRRs), which were up-regulated in *Xcf*-inoculated seeds (i.e., up-regulation of 15 annotated LRR related proteins), whereas the mitogen-activated protein kinases (MAPKs) and transcription factors (TF) of the basic leucine ZIPper (bZIP), TIFY and APETALA 2/ethylene responsive factor (AP2/ERF) classes were down-regulated. At 24 DAP, in parallel to the down-regulation of MAPKs known to be involved in defense signal transduction such as MAPKKK3, MAPK3 or MAPK4, we also identified down-regulation of defense related genes such as two encoding thaumatin pathogenesis-related (PR) proteins, five *JAZ* genes and one *JAR* gene involved in the jasmonic acid pathway, but also phyto alexin deficient 4 (*PAD4*), a central regulator of the salicylic acid pathway (Table S1). At 35 DAP, functional ontology enrichment detected four up-regulated categories related to peptidase/protease activities and transfer of carbon skeletons. At 42 DAP, only two up-regulated categories (chromatin regulation and calcium-permeable channel) were detected.

3.4 | sRNAs associated with *Xcf* seed colonization

To further characterize the molecular dialogue between the colonized seeds and *Xcf* and the changes in plant transcript expression we focused our analysis on sRNA changes between colonized and mock-treated seeds at two contrasted stages, at 24 DAP to decipher if transcriptome changes due to plant response to pathogen could be mediated by sRNAs and at 42 DAP to reveal if specific sRNA could be stored at seed maturity to mediate defense response at postgerminative stage. Following sequencing and mapping against the mature miRNA database (miRBase release 22), we observed a total of 255 and 112 mature miRNAs differentially expressed ($p < 0.05$) between *Xcf*-colonized and mock-treated seeds at 24 and 42 DAP, respectively. At 24 DAP, mature miRNA up-regulated in *Xcf*-colonized seeds belonged to six miRNA families (miR162, miR172, miR396, miR482, miR6478 and miR8175), while four miRNA families showed down-regulation (let7, miR21, miR2111 and miR482) (Table S2, Table 1). Similarly, at 42 DAP, we observed up-regulation of only one miRNA family (miR31) and down-regulation of two miRNA families (miR164 and miR451) (Table S2, Table 1). These data further confirmed that the molecular dialogue was more intense at early stages compared to later stages. Moreover, several miRNA families differentially regulated in *Xcf*-inoculated seeds were known to be involved in plant defense response such as miR482 (Shivaprasad et al., 2012), miR396 (Soto-Suárez et al., 2017) and miR172 (Holt et al., 2015). Known (from miRbase) and unknown (via the analysis of sRNA alignment clustering patterns) sRNAs were mapped to the genome using ShortStack version 4 and are available in the dedicated *P. vulgaris* Jbrowse (https://iris.angers.inrae.fr/pvulgaris_v2).

To reveal the potential response mediated by these miRNAs, we identified putative transcript targets using (i) psRNATarget predictive tool (Dai et al., 2018) combined with (ii) our generated transcriptomic data at these two stages (Table S2). To clarify, a transcript was considered as putative miRNA target if (i) its expectation (E) score from PsRNATarget was below 5 and if (ii) its expression was down-regulated when miRNA was up-regulated or inversely. Following these criteria, we identified between one to 11 putative miRNA target transcripts depending on miRNA families (Table 1). Out of these putative miRNA targets, we focused on genes known to be involved in either growth or defense related processes. Among miRNAs up-regulated at 24 DAP in *Xcf*-inoculated, we identified miR8175 that could down-regulate one of the key defense genes, *PAD4-like*, involved in the upstream defense pathway mediated by salicylic acid and others miR potentially affecting more generic defense genes such as a calcium-dependent-lipid-binding domain gene or phospholipase A1 (Table 1). At the opposite, in the *Xcf*-inoculated seeds, we observed down-regulation of miRNA families such as let7, miR21, miR2111 and miR482 that potentially enhanced expression of developmental/growth genes such as TOR-like, mediator 15 (MED15), MED13, NOC1/SWA2. At 42 DAP, only three miR families, miR31, miR451 and miR164, showed significant expression changes between *Xcf*-colonized and mock-treated seeds. An unique putative transcript target was identified associated with miR451, which encodes a UBP26-like protein potentially involved in the heterochromatin silencing at the end of the seed maturation (Luo et al., 2008). In conclusion, these results suggested that miRNA did mediate seed growth by silencing defense response at 24 DAP during early seed development. On the other hand at maturity, even if miR164 up-regulation was already shown to be involved in plant defense against fungi in cotton (*Gossypium hirsutum*) and *Populus tomentosa* (Chen, Wu, et al., 2021; Hu et al., 2020), in our susceptible host this miR was down-regulated at 42 DAP, which did not support the hypothesis that specific miRNA were accumulated in *Xcf*-inoculated seeds to prepare plant defense during germination. Interestingly, at 24 and 42 DAP, we observed that plant miRNA could support seed defense silencing probably due to the bacteria infection arsenal such as its T3Es activated early during seed development.

TABLE 1 Summary of differentially accumulated small RNAs (up- or down-regulated) detected at 24 and 42 DAP in *Xcf*-colonized *Phaseolus vulgaris* seeds with their putative target genes according to psRNAtarget.

	DEseq2			psRNAtarget combined with corresponding significant expression changes from RNA-seq data
Seed developmental stages	Up- or down-regulation in *Xcf*-colonized seeds	Mature miR	Variants	Putative targets using psRNAtarget (= miRNA potential target genes)
24 DAP	Up	miR162	a, b	Phvul.007G067800 (HSF), Phvul.008G055500 (TGD3), Phvul.006G176000 (trihelix DNA-binding), Phvul.008G114700 (Rab-GDF)
	Up	miR172	a, c, d, e, f, g, h, I, l	Phvul.009G014600 (cardiolipin deacylase), Phvul.001G212400 (RING-domain E3 ligase), Phvul.005G068800 (Probable E3 ubiquitin-protein ligase), Phvul.003G053000 (glycosyltransferase)
	Up	miR396	a, b, c, d, e, i	Phvul.009G246000 (SNF4-like), Phvul.001G229200 (pepsin-type protease), Phvul.002G026300 integrin-like protein), Phvul.003G154800 (HSP70)
	Up	miR482	3p, b-3p, d-3p	Phvul.011G149100 Transducin/WD40 repeat-like), Phvul.008G055500 (ATPase component TGD3 of TGD), Phvul.003G295800 (ATG2-like), Phvul.011G082700 (P-loop NTPase), Phvul.010G141400 (DOF1-like TF), Phvul.002G261500 (RNA polymerase regulatory protein)
	Up	miR6478	–	Phvul.003G155500 (component SR-alpha of SRP)
	Up	miR8175	–	Phvul.002G059000 (Phospholipase A1), Phvul.002G274500 (PAD4-like), Phvul.010G082300 (UDP-D-glucuronic acid 4-epimerase), Phvul.005G035400 (mRNA-splicing factor 18), Phvul.001G240600 (CaLB domain)
	Down	let7	a, c, d, f	Phvul.001G022700 (remorin-like), Phvul.003G119100 (calcium-dependent lipid-binding), Phvul.011G061600 (PTAC16 -like), Phvul.003G035400 (XYL1-like), Phvul.004G121666 (subunit of CF1 of ATP synthase), Phvul.008G163350 (cohesin cofactor (PDS5)), Phvul.011G050300 (protein kinase (PIKK) TOR-like), Phvul.003G050600 (catalytic protein (CER2)), Phvul.007G069900, Phvul.011G001200 (SAC1-like), Phvul.002G185150 (sodium:proton antiporter (SOS1))
	Down	miR21	a	Phvul.010G157900 (MED15-like), Phvul.007G191600 (CHR8-like)
	Down	miR2111	a, b, c, d, e, f, g, h, I, j, k, m, n, o	Phvul.001G269300 (MED13-like), Phvul.001G179300 (PGP1-like), Phvul.010G125200 (NOC1/SWA2-like), Phvul.007G168500 (Solute transport channels)
	Down	miR482	5p	Phvul.004G170000 (STT3-like), Phvul.010G125200 (NOC1/SWA2-like), Phvul.007G244066, Phvul.002G189700 (UPL1-like)
42 DAP	Up	miR31	–	–
	Down	miR451	a	Phvul.009G100000 (UBP26-like)
	Down	miR164	a, b, c, d, e, f, g, h, I, j, k	–

Abbreviations: DAP, day after pollination; miRNA, microRNA; RNA-seq, RNA sequencing; *Xcf*, *Xanthomonas citri* pv. *fuscans*.

3.5 | Seed methylome dynamics associated with *Xanthomonas* seed colonization

To better understand the plant defense response and the impact of the bacterial colonization during seed development, we analyzed the changes in the seed methylomes of mock-treated and *Xcf*-colonized bean seeds at seed maturity (42 DAP). Indeed, DNA methylation was already described as a relevant mechanism in defense priming and plant immunity (for review see, Deleris et al., 2016; Espinas et al., 2016). By focusing on the mature stage, we intended to capture the cumulative impact on DNA methylation of the bacterial colonization throughout seed development. The comparison of *Xcf*-colonized versus mock-treated seed samples revealed 954 DMRs, of which 61.95% were hypomethylated (loss of methylation due to bacterial colonization) and 38.05% hypermethylated (gain of methylation due to bacterial colonization) (Table S3). Not surprisingly, DMRs were predominantly localized on sequences containing transposable elements or repeats (74.1% of total DMRs), while 7.9% and 4.5% were located within gene and promoter sequences, respectively (Figure 3a). Regarding the methylation context, we mainly observed DMRs in the CHH (i.e., 481 DMRs) and CHG (i.e., 394 DMRs) contexts, while only 79 were related to the CG context. The complete list of the differentially methylated genes can be found in Table S3 and in the dedicated Jbrowse (https://iris.angers.inrae.fr/pvulgaris_v2).

We identified a total of 102 DMRs located within either coding ($n = 66$) or promoter regions ($n = 36$) of annotated genes, affecting 99 unique genes. Among coding sequences, 33 genes resulted in hypomethylation and 33 hypermethylation, while among promoter regions 27 genes were hypomethylated and 9 hypermethylated. To understand the role of genes differentially methylated in promoter and coding sequences at seed maturity, we compared with their changes in expression and did not observe any overlap with the DEGs between *Xcf*-colonized and noncolonized mature seeds, suggesting that differentially methylated regions did not regulate gene expression during seed development. To understand the potential role of these DMRs in the host-pathogen interaction, we looked at genes involved both in the germination and defense processes. First, from the data set generated from Narsai et al. (2017) during 10 early stages of *Arabidopsis* seed germination, we identified 21,015 genes showing a differential expression (adjusted $p < 1\%$ using ImpulseDE2) during germination process, therefore potentially involved in germination. By mapping *P. vulgaris* transcripts on Arabidopsis transcripts, we identified potential homologous transcripts in these two species and revealed a statistically significant enrichment (Figure 3b, Fisher's exact test $p < 2.2e{-}16$) of *P. vulgaris* genes displaying DMRs following pathogen colonization with those differentially expressed during germination. Indeed, out of the 90 homologous genes identified in *Arabidopsis* and displaying DMR, 78 were genes differentially expressed during germination (Figure 3b). Second, by analyzing the list of 99 unique genes displaying changes in methylation levels following bacterial colonization, we compiled a list of genes with putative roles in defense. We identified 17 genes, 10 hypomethylated and 7 hypermethylated following bacterial infection (Table 2). As example, we observed five LRR-related protein kinases, two PR proteins, and some genes identified as involved in immune response such as *PUB13-LIKE*, *CES11-LIKE* or *WRKY72* (complete list in Table S3). As it is known that changes in the methylation state of transposable regions can also spread to adjacent regions and regulate nearby gene expression (Ahmed et al., 2011), we extended our search to coding sequences that are 5 kb nearby DMRs located in transposable regions. This analysis detected additional 280 genes potentially associated with DMRs located in transposable regions (61.4% with hypomethylated regions and 38.6% with hypermethylated regions). Among these genes, we observed a subgroup coding for disease resistance proteins, with five additional putative TIR-NB-LRR proteins (*Phvul.004G105600*, *Phvul.004G100300*, *Phvul.010G026400*, *Phvul.010G027900*, *Phvul.010G028000*), three putative NB-ARC proteins (*Phvul.002G130300*, *Phvul.002G130400*, *Phvul.004G076100*) and four putative LRR kinases (*Phvul.008G164500*, *Phvul.008G164600*, *Phvul.005G162100*, *Phvul.005G162000*) (Table 2, Table S3 and in the dedicated Jbrowse). In total, we listed 17 DMRs nearby genes associated with defense processes (Table 2). A comparison between these two lists revealed that five genes encoding three *LRR related proteins (Phvul.008G164600, Phvul.005G162000 and Phvul.005G163000)*, *one TIR NBS LRR protein (Phvul.010G026400) and WRKY72 TF (Phvul.003G068700*, Phvul.010G062500*)*, displayed DMRs both within their gene sequences and in transposable elements located in proximal genomic regions.

To define the role of these DMRs present in defense genes, we analyzed gene expressions of six defense genes at postgermination stages (about 3 h after imbibition). Four genes encoding two LRRs (Phvul.005G162000 and Phvul.010G026400) and two WRKYs (Phvul.010G062500 and Phvul.003G068700) were selected based on their statistically contrasted methylation patterns due to *Xcf* colonization during seed development. Two other WRKYs (Phvul.006G07460 and Phvul.008G251300) were selected due to the absence of DMRs in their surrounding genomic regions following *Xcf* colonization in seeds. Expression profiles of these genes were analyzed at the early germination stage (about 3 h after imbibition) on mock-treated seeds during development then inoculated with water at germination stage (mock–mock experiment), on mock-treated seeds during development then inoculated with 10^7 of *Xcf* CFU mL^{-1} at germination stage (mock–*Xcf* experiment), on Xcf-colonized seeds during development then inoculated with 10^7 of *Xcf* CFU mL^{-1} at germination stage (*Xcf*–*Xcf* experiment) (Figure 3c). Regarding the defense genes with no change in methylation pattern due to *Xcf* colonization, we did not observe any change in expression between the three treatments (i.e., mock–mock, mock–*Xcf* and *Xcf*–*Xcf*). In contrast, two (Phvul.003G068700 and Phvul.010G026400) of the four defense genes displaying changes in their methylation patterns showed statistically different expression profiles with increases of expressions when the pathogen was inoculated during germination and decreases of expressions when the pathogen was inoculated during germination of seeds already colonized by *Xcf* during seed development (i.e., when pre-exposed to the pathogen before germination) (Figure 3c). The expression patterns of the two other defense genes (Phvul.005G162000 and Phvul.010G062500)

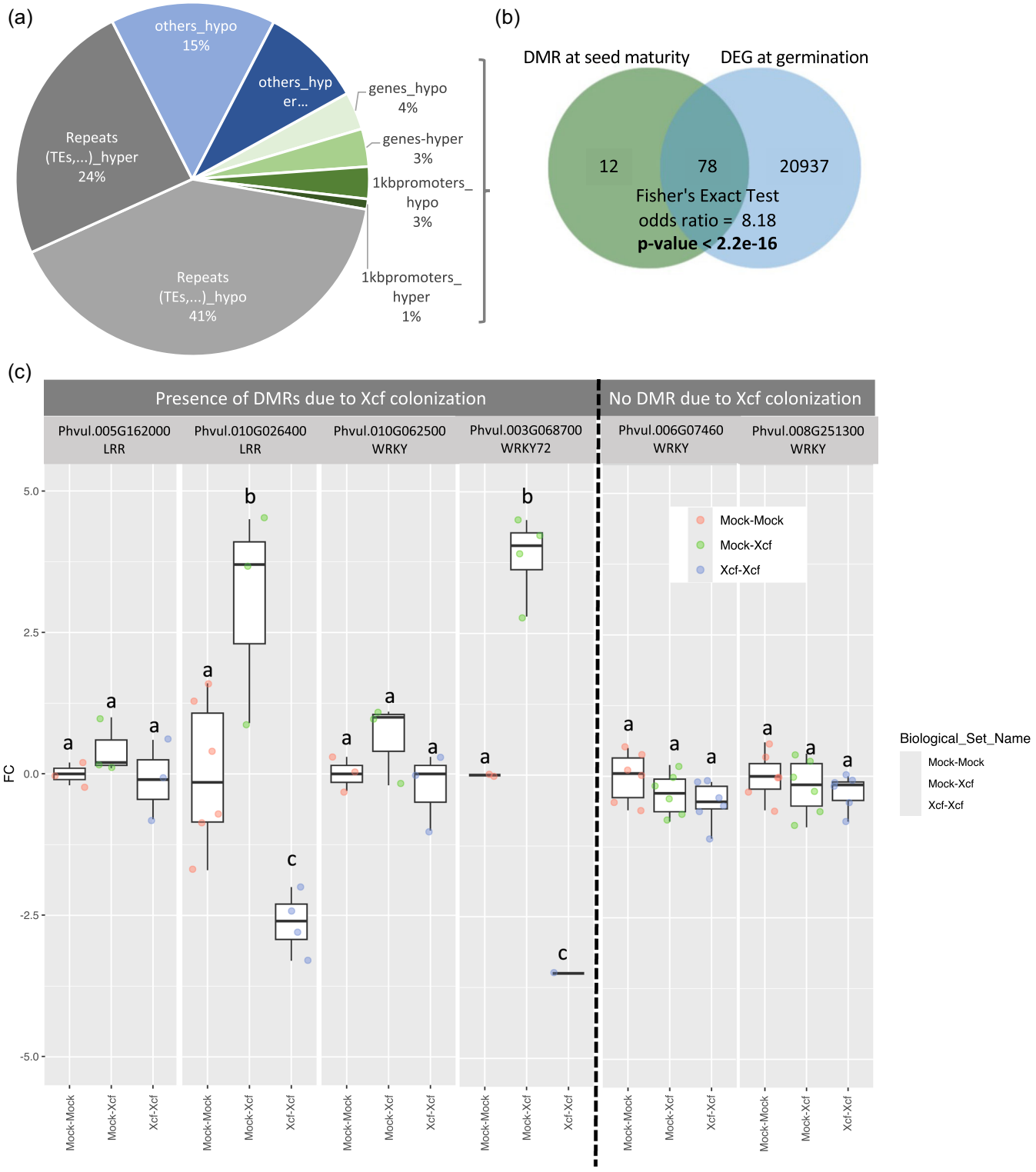

FIGURE 3 (See caption on next page).

followed the same tendency but no statistical changes were observed (Figure 3c). This result clearly showed that the presence of *Xcf* during seed development induced plant defense silencing during germination via changes in DNA methylation regarding a LRR (Phvul.010G026400) and a WRKY72 (Phvul.003G068700) genes. Interestingly, we observed colocalization between small interfering RNA (siRNA) clusters identified at 24 and 42 DAP using Shortstack and differentially methylated regions in several genes including the ones analyzed above (e.g., WRKY33, Phvul.010G062500; LRR, Phvul.005G162000; LRR, *Phvul.008G164600*), suggesting that

methylation changes could result from production of siRNA produced from 24 DAP in *Xcf*-colonized seeds, as part of the pathogen arsenal to improve its transmission to seedling.

Together, these results suggested that DMRs located nearby gene sequences due to the presence of *Xcf* could result from the early seed developmental stage to silence the plant immune response to *Xcf* during germination. In other word, pathogen-specific DNA methylations occurring during seed development could serve as plant defense silencing to repress plant immune response during the germination process, and increase chances of pathogen transmission to seedlings.

4 | DISCUSSION

Seeds are essential components of plants fitness and represent an important means of pathogen dispersion. To date, seed–pathogen interactions have been understudied at the molecular level, with, to our knowledge, only one plant-orientated study describing the transcriptomic response of *Medicago truncatula* seeds to bacterial pathogens of the Xanthomonadaceae family (Terrasson et al., 2015). We thus attempted to mitigate this knowledge gap by describing the molecular dialogue between common bean seeds and *Xcf* in conditions that seed bacterial transmission was asymptomatic. A first central result regarding this interaction is that *Xcf* was able to colonize seeds without major impact on seed physiology parameters, which was reflected by similar dry weights and water contents in mock treated- and colonized-seeds (Figure 1). Consequently, we could not observe any obvious morphological changes in *Xcf*-colonized seeds compared to mock treated samples. Such findings indicate that asymptomatic *Xcf* colonization does not impact seed development or alter seed growth. This is consistent with previous report in *M. truncatula* during compatible interaction with *Xanthomonas euvesicatoria* pv. *alfalfae*, while incompatible interaction with *Xcc* resulted in developmental defects alongside a strong activation of defense pathways (Terrasson et al., 2015).

To look into molecular dialogue, transcriptomic changes were assessed using dual RNA-seq, which implies that we profiled both bacterial and plant transcripts during seed development generating the first dual transcriptomic analysis of a seed–pathogen interaction ever made. Profiling of bacterial transcripts represented the main challenge we faced due to the low concentration of bacterial cells within seeds. In this study, we successfully achieved this technological breakthrough by an enrichment step of bacterial transcripts using an RNA capture technology (Agilent). Our study revealed that *Xcf* and common bean seeds establish an intense molecular dialogue at the early stages of seed development that appears to become less intense as seed maturity approaches (Figure 2).

On the pathogen side, the up-regulation of the T3SS genes and cognate effectors observed in the early stages in comparison with 42 DAP suggests they could play a role in the host defense silencing during the early step of seed colonization (Büttner, 2016). Indeed, Xanthomonad's T3SS and T3Es are known to play two crucial roles in allowing efficient bacterial seed transmission (Darsonval et al., 2008) and in suppressing plant innate immunity and modulate plant pathways for the benefits of the bacteria (Büttner, 2016). Interestingly, down-regulated categories at early stages include basic biological processes such as translation, protein turnover and DNA replication. This might suggest that *Xcf* multiplication is hampered, consistently with the observation that number of *Xcf* cells in seeds does not increase significantly throughout seed developmental stages (Figure 1c). Fewer functional categories were enriched at 35 DAP (Figure 2d). The up-regulated ones (four out of five) included peptidases, glycosylases and methyl transferases. Such functions can be associated with both suppression of defense (peptidases, Figaj et al., 2019) and cell wall remodelling, which could help bacterial colonization of seed tissues, with no detectable impact on the seed physiology and morphology, although more subtle microscopical effects cannot be excluded (Figure 1).

On the host side, we also observed intense gene expression changes at early seed developmental stage (24 DAP) in comparison to later ones, concomitantly with the expression of bacterial secretion proteins. We observed an enrichment of up-regulated LRR protein kinases (two categories out of six, LRR class VIII and class Xb), which are known to have prominent roles in microbe perception and defense activation in nonseed tissues such as leaves (Chakraborty

FIGURE 3 Summary and transcriptional impacts of changes in methylation patterns between *Xcf*-colonized and uncolonized seeds at 42 DAP. (a) Pie chart illustrating the repartition of differentially methylated regions (DMRs) following *Xcf* colonization on *Phaseolus vulgaris* genome at 42 DAP. Numbers of hypo- and hypermethylated regions (DMR) considering *Xcf*-inoculated versus mock-treated seeds are indicated. Genomic regions (promoters, genes, repeats, TE) were identified from the genome annotation file. (b) Venn diagram illustrating the overlap between gene sequences containing DMRs at 42 DAP and differentially expressed genes (DEGs) during germination (see details in text). (c) Relative expression profiles of genes potentially involved in defense during early germination stage (about 3 h after imbibition) and displaying (or not) DMR due to the *Xcf* colonization in developing seeds. Three conditions were applied: mock–mock corresponding to mock-treated seeds during development then inoculated with water at germination stage, mock–*Xcf* corresponding to mock-treated seeds during development then inoculated with 10^7 of *Xcf* CFU mL^{-1} at germination stage and *Xcf*–*Xcf* corresponding to *Xcf*-colonized seeds during development then inoculated with 10^7 of *Xcf* CFU mL^{-1} at germination stage. Expression values were obtained using the $\Delta\Delta C_t$ method by normalizing first against the geometric mean of housekeeping genes then against the mock–mock treatment, then values were Log_2-transformed to visualize fold changes (FC). Statistical test were assessed using one-way ANOVA followed by post hoc Tukey's test. ANOVA, analysis of variance; CFU, colony-forming unit; DAP, day after pollination; TE, transposable element; *Xcf*, *Xanthomonas citri* pv. *fuscans*. [Color figure can be viewed at wileyonlinelibrary.com]

TABLE 2 List of differentially methylated regions located in defense-associated genes in of *Phaseolus vulgaris* seeds following *Xcf* colonization at 42 DAP.

P. vulgaris locus ID	Location	*P. vulgaris* annotation	*Arabidopsis thaliana* putative ortholog	*A. thaliana* symbol	*A. thaliana* annotation	Methylation FC (*Xcf* vs. H_2O)	Gain or loss of methylation in *Xcf*-treated seeds
Phvul.001G233000	Gene	Protein kinase (SD-1)	AT3G16030	CES101	Lectin protein kinase family protein	0.52	Loss
Phvul.002G125500	Within 5 kb	Not annotated	AT5G08315		Defensin-like (DEFL) family protein		Loss
Phvul.002G130300	Within 5 kb	Not annotated	AT3G14470		NB-ARC domain-containing disease resistance protein		Gain
Phvul.002G130400	Within 5 kb	Not annotated	AT3G14470		NB-ARC domain-containing disease resistance protein		Gain
Phvul.003G021700	1 kbProm	Transferase transferring phosphorus-containing group	AT1G70740		Protein kinase superfamily protein	0.51	Loss
Phvul.003G040300	1 kbProm	6-deoxocastasterone 6-oxidase	AT3G30180	BR6OX2	Brassinosteroid-6-oxidase 2	2.98	Gain
Phvul.003G056900	Within 5 kb	Systemic acquired resistance regulator protein (SNI1)	AT4G18470	SNI1	Negative regulator of systemic acquired resistance (SNI1)		Loss
Phvul.003G068700	Gene	Transcription factor (WRKY)	AT5G15130	WRKY72	WRKY DNA-binding protein 72	4.30	Gain
Phvul.003G068700	Within 5 kb	Transcription factor (WRKY)	AT5G15130	WRKY72	WRKY DNA-binding protein 72		Gain
Phvul.003G175700	Gene	DRB4-DRB7.1 regulator complex.component DRB7	AT5G20320	DCL4	Dicer-like 4	0.37	Loss
Phvul.004G076100	Within 5 kb	Not annotated	AT3G14470		NB-ARC domain-containing disease resistance protein		Loss
Phvul.004G105600	Within 5 kb	Not annotated	AT2G34930		Disease resistance family protein/LRR family protein		Loss
Phvul.005G162000	1kbProm	Transferase transferring phosphorus-containing group	AT4G29990		Leucine-rich repeat transmembrane protein kinase protein	2.36	Gain
Phvul.005G162000	Within 5 kb	Transferase transferring phosphorus-containing group	AT4G29990		Leucine-rich repeat transmembrane protein kinase protein		Gain
Phvul.005G162100	Within 5 kb	Transferase transferring phosphorus-containing group	AT1G51800		Leucine-rich repeat protein kinase family protein		Gain
Phvul.005G163000	Gene	Transferase transferring phosphorus-containing group	AT4G29990		Leucine-rich repeat transmembrane protein kinase protein	0.38	Loss
Phvul.005G163000	Within 5 kb	Transferase transferring phosphorus-containing group	AT4G29990		Leucine-rich repeat transmembrane protein kinase protein		Loss

TABLE 2 (Continued)

P. vulgaris locus ID	Location	*P. vulgaris* annotation	*Arabidopsis thaliana* putative ortholog	*A. thaliana* symbol	*A. thaliana* annotation	Methylation FC (*Xcf* vs. H_2O)	Gain or loss of methylation in *Xcf*-treated seeds
Phvul.006G006800	Gene	TKL protein kinase superfamily.protein kinase (DUF26)	AT4G05200	CRK25	Cysteine-rich RLK (RECEPTOR-like protein kinase) 25	0.18	Loss
Phvul.006G033200	Gene	Not annotated	AT5G38280	PR5K	PR5-like receptor kinase	0.52	Loss
Phvul.007G187700	1 kbProm	Not annotated	AT3G04720	PR4	Pathogenesis-related 4	0.37	Loss
Phvul.007G241200	Gene	Transcription factor (MYB-related)	AT5G47390		myb-like transcription factor family protein	3.25	Gain
Phvul.007G241200	Within 5 kb	Transcription factor (MYB-related)	AT5G47390		myb-like transcription factor family protein		Gain
Phvul.007G241300	Within 5 kb	Ser/Thr protein kinase	AT1G50240	FU	Protein kinase family protein with ARM repeat domain		Gain
Phvul.008G164500	Within 5 kb	Transferase transferring phosphorus-containing group	AT3G21340		Leucine-rich repeat protein kinase family protein		Loss
Phvul.008G164600	Gene	Not annotated	AT1G05700		Leucine-rich repeat transmembrane protein kinase protein	0.45	Loss
Phvul.008G164600	Within 5 kb	Not annotated	AT1G05700		Leucine-rich repeat transmembrane protein kinase protein		Loss
Phvul.008G228714	Gene	S8-class protease (subtilisin) families.protease (SBT4)	AT3G46850		Subtilase family protein	4.29	Gain
Phvul.008G229400	Gene	S8-class protease (subtilisin) families.protease (SBT4)	AT5G59100		Subtilisin-like serine endopeptidase family protein	0.48	Loss
Phvul.010G026400	1 kbProm	Effector receptor (NLR)	AT5G36930		Disease resistance protein (TIR-NBS-LRR class) family	0.35	Loss
Phvul.010G026400	Within 5 kb	Effector receptor (NLR)	AT5G36930		Disease resistance protein (TIR-NBS-LRR class) family		Loss
Phvul.010G027900	Within 5 kb	Effector receptor (NLR)	AT5G36930		Disease resistance protein (TIR-NBS-LRR class) family		Loss
Phvul.010G028000	Within 5 kb	Effector receptor (NLR)	AT5G36930		Disease resistance protein (TIR-NBS-LRR class) family		Loss
Phvul.010G062500	Within 5 kb	WRKY33-dependent plant immunity transcription factor	AT2G38470	WRKY33	WRKY DNA-binding protein 33		Loss
Phvul.011G064700	Gene	U-box E3 ligase activities.E3 ubiquitin ligase (PUB)	AT3G46510	PUB13	Plant U-box 13	4.06	Gain

(Continues)

TABLE 2 (Continued)

P. vulgaris locus ID	Location	*P. vulgaris* annotation	*Arabidopsis thaliana* putative ortholog	*A. thaliana* symbol	*A. thaliana* annotation	Methylation FC (*Xcf* vs. H_2O)	Gain or loss of methylation in *Xcf*-treated seeds
Phvul.011G108300	Gene	Transferase transferring phosphorus-containing group	AT1G29730		Leucine-rich repeat transmembrane protein kinase	2.32	Gain
Phvul.011G176100	Gene	Transferase transferring one-carbon group	AT3G11480	BSMT1	S-adenosyl-L-methionine-dependent methyltransferases family protein	2.56	Gain

Note: DMRs were located in promoter or gene sequences, but also in transposable elements located within 5 kb of genic regions. The *P. vulgaris* annotation column was filled according to the *P. vulgaris* genome (v2.1). The location indicates whether the region is localized in a coding region (gene) or in the promoter (1 kbprom) or in TE within 5 kb of genic regions (within 5 kb). The putative ortholog was assigned as best hit based on sequence similarity in the *Arabidopsis* genome (v.11). The 'gain or loss' column shows whether the differentially methylated region associated with the corresponding *P. vulgaris* gene is hypo- (loss) or hypermethylated (gain) in response to *Xcf* colonization at 42 DAP. FC ratios are not indicated for DMRs within 5 kb of genic regions because they correspond to multiple DMRs.

Abbreviations: DAP, day after pollination; DMR, differentially methylated region; FC, fold change of methylation between *Xcf*- versus H_2O-treated seeds; TE, transposable element; *Xcf*, *Xanthomonas citri* pv. *fuscans*.

et al., 2019), suggesting that the host may be able to recognize the pathogen. On the other hand, RNA-seq data highlighted a down-regulation of gene categories with well characterized roles in the transduction of defense signalling pathways, including MAPKs (such as MAPKKK3, MAPK3 or MAPK4) and TFs of the bZIP, TIFY, and AP2/ERF families (Bai et al., 2011; Bethke et al., 2009; Noman et al., 2017; Tintor et al., 2013). In line with this, we observed down-regulation of TF families known to have wider functions in plant stress signalling, such as the TUB or TUBBY-like proteins and the heat shock transcription factor (HSF), as well as genes encoding PR proteins, including *JAZ* and *JAR* genes involved in the jasmonic acid pathway, and *PAD4* involved in the salicylic acid pathway. Such data suggest that even the transduction components of the defense pathway are inhibited, potentially due to the bacterial T3E, ultimately avoiding a defense response.

Similar to transcriptomic data, changes in the expression of sRNAs at 24 DAP and 42 DAP were consistent with the silencing of downstream defense gene response. Indeed, analysis of the differentially expressed miRNA at 24 DAP and their putative target genes suggests a growth/defense trade-off mechanism in favour of growth in *Xcf*-inoculated seeds, with down-regulation of defense-associated transcripts (e.g., a putative ortholog of PAD4 (*Phvul002G274500*, involved in salicylic acid signalling in *Arabidopsis;* Pruitt et al., 2021), a pepsin-type protease (*Phvul001G229200*)) and up-regulation of development-associated transcripts (e.g., TOR-like (*Phvul011G050300*) and MED15 (*Phvul010G157900*, required for correct embryogenesis in *Arabidopsis;* Kim et al., 2016). Interestingly, a heat shock protein (HSP70, *Phvul003G154800*) was detected as down-regulated genes at 24 DAP in *Xcf*-inoculated seeds and potential target of miR396, which completed the observed down-regulation of HSF and smallHSP from our infected host transcriptome data (Figure 2d). Recently it was shown that HSPs are the most represented family among the down-regulated DEGs in leaf in a resistant common bean genotype towards CBB (caused by *Xcf* and *Xanthomonas phaseoli* pv. *phaseoli*) in comparison to a susceptible one (Foucher et al., 2020). On the other hand, data obtained at 42 DAP revealed only down-regulation of one miRNA family miR451, potentially regulating the up-regulation of its predicted target gene (*Phvul009G100000*) (Table 1). Its *Arabidopsis* protein homolog (AT3G49600.1) deubiquitinates the histone H2B and is required for heterochromatin silencing during seed development (Luo et al., 2008). It is worth noting that chromatin reorganization processes due to histone modifications are among the categories enriched at 42 DAP (Figure 2d), therefore suggesting that epigenetic regulation is a relevant component of the seed–pathogen molecular dialogue at this stage, potentially acting as priming for postgermination phase. Globally, the transcriptomic response of the susceptible host plant suggests that developing seeds are able to perceive the pathogen, and that defense responses might be largely inhibited by the bacterial T3SS arsenal. Consistent with suppression of the plant defense, up-regulation of photosynthesis and down-regulation of cell wall organization enzymes (Figure 2d) was also previously observed in leaves of susceptible common bean plants upon infection (Foucher et al., 2020). On the other

hand, down-regulation of HSP and HSF, and AP2/ERF TFs (Figure 2d) were the hallmark of resistant plants. This suggests that a balance between susceptibility and resistance exist in *Xcf-colonized* seeds, which could explain why, despite active bacterial colonization, the seeds were asymptomatic and presented no obvious physiological impact.

In this study, we also revealed a relevant role for epigenetic modifications in *Xcf*-colonized seeds, which act as plant defense silencing for the germination phase, explaining the efficient transmission of *Xcf* to seedlings. First, we highlighted a statistically significant enrichment between DMRs-containing genes and DEGs during germination (Figure 3b). This suggests that the DMR-containing genes following bacterial infection detected in this study may serve during the germination process. Moreover, the seed host methylome analysis at 42 DAP revealed significant changes in methylation status in 826 different genomic regions, affecting a total of 99 different genes, which did not display any change in gene expression during seed maturation. Of these, 17 can be associated with defense processes in a relatively straightforward manner (Table 2). As hypomethylation of defense genes has been widely associated with increased resistance to biotic stress (Annacondia et al., 2021; Dowen et al., 2012), the hypomethylated genes of this list (10 out of 17) can be considered as candidates for epigenetic-dependent defense priming such as *WRKY72* gene (Phvul.003G068700). It was tempting to speculate that such modifications promote defense priming to prepare the host to face a novel pathogen assault after germination. The concept of defense priming postulates that plants conserve the memory of previous encounters with pathogens by preparing their defense networks to respond more rapidly and strongly to a future aggression (Martinez-Medina et al., 2016). Enhanced chromatin access to defense genes through hypomethylation is one of the best characterized mechanisms in this sense (Hannan Parker et al., 2022). Furthermore, epigenetic defense priming can be transmitted to the next generations (Slaughter et al., 2012). This would be consistent with a scenario where *Xcf* colonization does not directly induce defense gene activation in common bean seeds, but rather triggers a primed state that prepare defense networks for the moment when the pathogen will again become virulent (after germination). However, expression profiling of four defense genes showing DMRs in their promoter regions following *Xcf* colonization suggested the opposite effects of these modifications, a plant defense silencing. Indeed, out of the four genes, two of them clearly showed silencing of their expression during germination following *Xcf* colonization during seed development. The two other ones did not display any significant expression changes but a tendency that could suggest they act at different timing of germination or postgermination phases (Figure 4). A detailed kinetics of the transcriptome and epigenome of the bean–*Xcf* interaction during the germination process would be a promising future research direction to conclude if these epigenetic

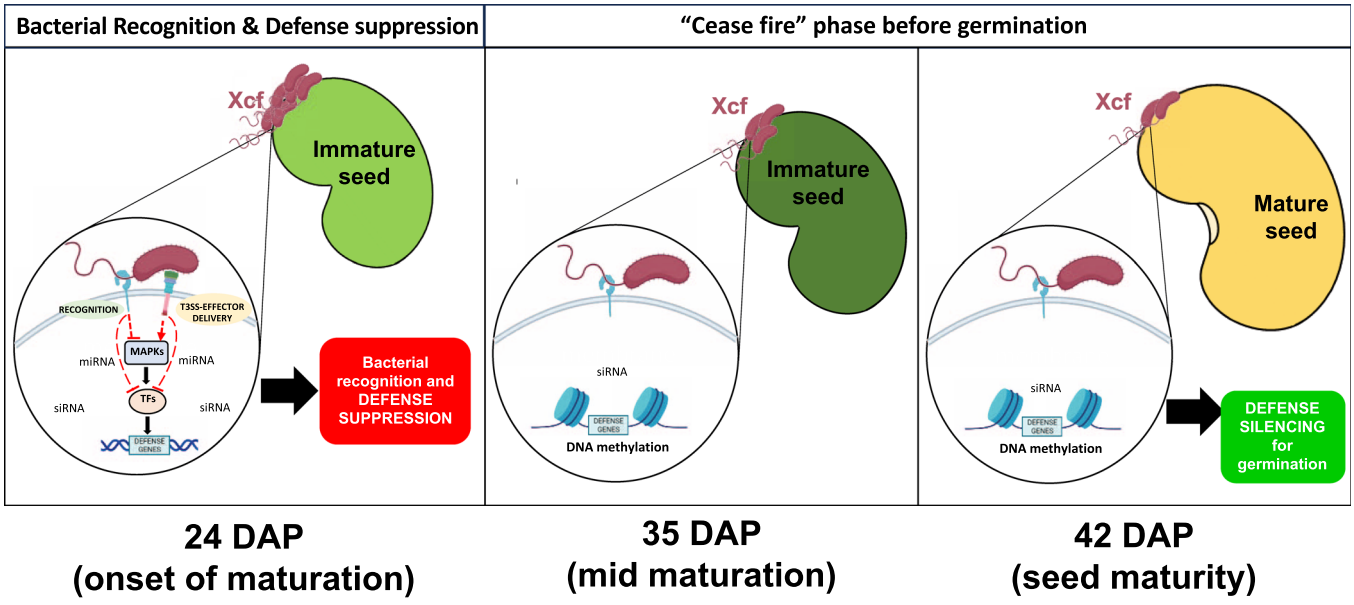

FIGURE 4 Schematic model of the *Xcf*-bean seed dialogue. Left panel: at early seed development stages (24 DAP), *Xcf* is recognized by the host. Despite the bacterial recognition, defense transduction pathways based on MAP kinases cascades (MAPKs) and transcription factors (TFs) activation are suppressed in seeds, thus failing to induce a defense reaction. Red dotted lines with flat end indicate hypothetical inhibition. Middle panel: at 35 DAP, both the bacterial pathogen and the host plant are still transcriptionally active. Bacterial populations continue to grow, but the T3SS is no longer active, suggesting that the bacteria lowered its weapons, keeping the seed alive and healthy. Right panel: at seed maturation (42 DAP), the dialogue between *Xcf* and seed is much less detectable in comparison to earlier stages but we observed changes in DNA methylation due to *Xcf* colonization that colocalized with clusters of siRNA. Changes in the methylation status of genes potentially involved in defense response could silence plant defense gene at the germination stage, explaining the efficient transmission of the pathogen to seedling (see text for more details). DAP, day after pollination; miRNA, microRNA; siRNA, small interfering RNA; T3SS, type 3 secretion system; *Xcf*, *Xanthomonas citri* pv. *fuscans*. [Color figure can be viewed at wileyonlinelibrary.com]

modifications are exclusively in favour of the pathogen (i.e., plant defense silencing) to efficiently colonize seedling or if the situation is more contrasted with some modifications to enhance plant immune response (i.e., plant defense priming).

All together, these results indicate that the molecular mechanisms involved in the pathogen-seed dialogue change radically across the developmental stages for both the host and the pathogen side, potentially suggesting the existence of distinct phases in the considered seed–pathogen interaction. It would be interesting to explore whether such patterns take place in other seed–pathogen interactions. By summing our physiological and molecular observations, with the previous findings of Terrasson et al. (2015), we can propose a model where the recognition of a host-specific pathogen at the early stages of seed development fails to trigger seed defense activation, as if the presence of the pathogen was 'accepted' by the host. Even if we cannot define whether this suppression is caused by the pathogen or by the host, two *Xanthomonas* studies would support the role of bacterial T3SS in host defense silencing. Darsonval et al. (2008) showed the essential role of T3E for an efficient seed colonization in the *Xcf*–bean seed interaction and Terrasson et al. (2015) showed that *X. euvesicatoria* pv. *Alfalfae* was able to silence some defense genes in a compatible interaction, but not in incompatible one. In any case, the result is a situation where the seed develops normally without any obvious fitness costs associated to an eventual defense activation, while the host-specific pathogen displays a nonaggressive behaviour throughout all the seed development and limits its proliferation (Figure 4). Such 'ceasefire' scenario might be advantageous for both parts: the seed is able to reach maturity, which would potentially be beneficial for the pathogen as well by allowing it to infect the future germinated seedling, therefore giving it access to nourishment and facilitating its dispersal. Finally, germination and postgermination stages of mock-treated and *Xcf*-colonized seeds are also to consider as another distinct phase, highly influenced by the *Xcf* colonization during seed development. Indeed, recent data from the compatible interaction *Alternaria brassisicolae*–*Arabidopsis*, used as seed transmission model, showed that host defense pathways are subjected to drastic changes during the different phases of germination and postgermination processes (Ortega-Cuadros et al., 2022). It would be interesting to explore whether such rearrangements take place in other compatible interactions such as *Xcf*–bean and if a link with epigenetic modifications exists.

To summarize, the present study adds novel elements to the current knowledge gap of seed–pathogen interactions. The dual transcriptomic analysis allowed for the first time to describe the molecular dialogue from both host and pathogen sides, while methylome and sRNAs profiling added further indications on the potential regulatory mechanisms and the genes involved. A dedicated Jbrowse containing all these generated data will serve as baseline tool for the scientific communities and will be enriched by future related studies. A general conclusion that we can draw is that seeds have primarily an active role in this interaction at early seed maturation stage, contrary to the widely diffused assumption considering seeds as passive carriers of microbes (Dutta et al., 2014). As the role of seedborne pathogens in causing yield losses receives relatively little attention, we hope that the present study can stimulate novel research efforts in this sense to shed light on the many obscure points still shrouding seed–pathogen interactions.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study have been deposited in NCBI Gene Expression Omnibus and are accessible through GEO Super Series accession number GSE227421 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE227421) or individually through GEO accession numbers GSE227386 (bacterial RNA-seq, https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE227386), GSE226918 (plant RNA-seq, https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE226918), GSE226919 (plant methylome, https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE226919) and GSE226920 (sRNA-Seq, https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE226920).

ORCID

Armelle Darrasse http://orcid.org/0000-0002-9334-5862
Łukasz Paweł Tarkowski http://orcid.org/0000-0002-5419-6606
Nicolas W. G. Chen http://orcid.org/0000-0002-7528-4656
Matthieu Barret http://orcid.org/0000-0002-7633-8476
Jerome Verdier http://orcid.org/0000-0003-3039-2159

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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