DNA Methylation of ABC Transporters Differs in Native and Non-native Populations of *Conyza canadensis* L.

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While differences in the methylation patterns of ABC transporters under different environmental conditions and their role in plant growth, development, and response to biotic and abiotic stresses are well documented, less is known about the variation in the methylation patterns of ABC transporters in plant species in the native and non-native ranges. In this study, we present the results of differences in methylation of ABC transporters of *Conyza canadensis* L. in its native (North America) and non-native (Kashmir Himalaya) ranges. Our data show that ABC transporter genes have reduced DNA methylation in Kashmir Himalaya than in North America. Furthermore, in the non-native range of Kashmir Himalaya, we found that ABC transporter genes have enriched RNA Pol-II binding and reduced nucleosome occupancy, both hallmarks of transcriptional activity. Taken together, our study showed differential DNA methylation in the ABC transporter genes in the native range of North America and non-native range of Kashmir Himalaya in *Conyza canadensis* and that the reduced DNA methylation and increased RNA Pol-II binding is one of the possible mechanisms through which this species in the non-native range of Kashmir Himalaya may show greater gene expression of ABC transporter genes. This increased ABC transporter gene expression may help the plant to grow in different environmental conditions in the non-native range. Furthermore, this study could pave way for more studies to better explain the enigmatic plant invasions of *C. canadensis* in the non-native range of Kashmir Himalaya.

Keywords: ABC transporters, chromatin, epigenetics, DNA methylation, plant invasion, phenotypic plasticity, adaptation

INTRODUCTION

DNA methylation, one of the most common epigenetic modifications, contributes to the overall growth, development, and tolerance to both biotic and abiotic factors (Alonso et al., 2018; Agarwal et al., 2020; Eriksson et al., 2020; Gallego-Bartolom, 2020; Liu and He, 2020; Kumar and Mohapatra, 2021). DNA methylation and other epigenetic modifications are now known to adjust phenotypes instantaneously and/or generate new phenotypes in a reversibly heritable manner (Flatscher et al., 2012; Miryeganeh and Saze, 2019). These heritable epigenetic variations could directly or indirectly

influence the course of evolution in plants, as they can affect the processes of adaptation (Rapp and Wendel, 2005; Jablonka and Raz, 2009).

Epigenetic processes, including DNA methylation, have emerged as important mechanisms contributing to adaptation and spread of alien plants in new habitats in the non-native ranges (Perez et al., 2015), despite several bottlenecks, such as low propagule pressure, low genetic variability, climate incompatibility, inadequate or inappropriate resources, or small population size (Banerjee et al., 2019). *Conyza canadensis,* commonly called as horseweed, has successfully adapted to several non-native habitats across the world and is considered a highly invasive problematic annual weed species of many agronomic crops in about 70 countries (Travlos and Chachalis, 2010; Moretti et al., 2017). This species has a very prolific growth and is reported to produce up to 200,000 seeds, which are easily dispersed by wind to infest new territories (Dauer et al., 2007). To control this weed species, commercial herbicides are in use for a long time but *Conyza* species have developed resistance against these herbicides, particularly glyphosate (Moretti et al., 2017). Vacuolar sequestration of herbicides has been identified as the predominant resistance mechanism that neutralizes its activity mediated through tonoplast localized ABC transporters (Peng et al., 2010; Douglas and Gaines, 2014; Ge et al., 2014). ABC transporter genes such as M10 and M11 are highly expressed in herbicide-resistant *Conyza* species (Peng et al., 2010; Nol et al., 2012; Moretti et al., 2017). Owing to environmental influence, there is a decreased expression of ABC transporters such as M10 and M11 under conditions of low temperature resulting in a lack of glyphosate resistance (Tani et al., 2016). The possible mechanisms of ABC transporter gene expression in invasive populations could be DNA methylation-induced alterations in chromatin structure, which, in turn, may be responsible for higher adaptability and invasiveness. Keeping in view the differential methylation patterns across various habitats and the potential role played by epigenetic diversity in influencing the invasive traits of a plant (Zhang et al., 2016; Banerjee et al., 2019), we hypothesized differential epigenetic signatures of *C. canadensis* in its native range of North America and non-native range of Kashmir Himalaya. The study, the first of its kind on *C. canadensis*, is likely to open new vistas of investigating the invasiveness of alien species in the non-native ranges, which is a prerequisite for their better management.

MATERIALS AND METHODS

Sampling of Leaf Material
Leaf samples at the flowering stage were collected from both the native range of North America and the non-native range of Kashmir Himalaya from 5 different sites. Each sample was collected along with three biological replicates and three technical replicates. The sites, namely, Kangan, Gulmarg, Budgam, KUBG1 (Kashmir University botanical garden), and KUBG2, were selected for the collection of non-native leaf samples of Kashmir Himalaya, while Turah, Clinton, Clarke, Butte, and Lolo were selected for the native leaf samples of North America.

S.No.	Site name	Geo-coordinates	Part collected	Sampling time
United States				
1.	Turah		Leaf	Flowering stage
2.	Clarke	46° 40' 8'' N 111° 44' 0'' W	Leaf	Flowering stage
3.	Butte	46°0'23''N 112°31'47''W	Leaf	Flowering stage
4.	Lolo	46°45'55''N 114°5'9''W	Leaf	Flowering stage
5.	Clinton	46°46'33''N 113°42'54''W	Leaf	Flowering stage
Kashmir				
1.	KUBG1	34°07'57''N 74°50'15''E	Leaf	Flowering stage
2.	KUBG2	34.1589887°N 74.8316992°E	Leaf	Flowering stage
3.	Gulmarg	34.05°N 74.38°E	Leaf	Flowering stage
4.	Kangan	34.263°N 74.903°E	Leaf	Flowering stage
5.	Budgam	34°1'12''N 74°46'48''E	Leaf	Flowering stage

Chromatin Extraction Followed by Methylated DNA Immunoprecipitation
Chromatin was extracted from the leaf samples of both the native range of North America and the non-native range of Kashmir Himalaya of *C. canadensis*. After quantification, approximately 6 μg of DNA was taken for sonication (20% amplitude, pulse: 30 s, and time: 3 min). Samples were normalized and incubated at 4°C using an antimethyl cytosine antibody overnight with end-to-end rotation. For pull-down, equilibrated Dynabeads were added to each sample. Then samples were treated with proteinase K and glycogen, incubated at 55°C, 1,200 rpm overnight (Fabio et al., 2009). DNA was extracted using PCI (phenol:chloroform:isoamyl) and resuspended in 1 × TE buffer, followed by quantitative PCR (qPCR).

Chromatin Immunoprecipitation Assay
Approximately 2 g leaf samples of *C. canadensis* from both the native range of North America and the non-native range of Kashmir Himalaya were taken and submerged in 37% formaldehyde and 2.5 M glycine for crosslinking of tissue using a vacuum infiltrator (Saleh et al., 2008). The tissue was then washed two times with double distilled water and then resuspended in extraction buffer 1 (0.4 M sucrose, 10 mM Tris-HCl, 5 mM βmerc, 0.1 mM PMSF, PIC) and filtered through Mira cloth (Merck Millipore, Burlington, MA, United States). After centrifugation for 20 min at 5,000 rpm, the pellet was resuspended in 1 ml of extraction buffer 2 (0.25 M sucrose, 10 mM Tris-HCl, 10 mM MgCl$_2$, 1% Triton x-100, 5 mM βmerc, 0.1 mM PMSF, and PIC) followed by 300 μl of extraction buffer 3 (1.7 M sucrose, 10 mM Tris-HCl, 0.15% Triton x-100, 2 mM MgCl$_2$, 5 mM βmerc, 0.1 mM PMSF, and PIC). Isolated chromatin was resuspended in nuclei lysis buffer (50 mM Tris-HCl, 10 mM EDTA, 1% SDS, PMSF, and PIC) followed by sonication (30 s ON, 30 s OFF, 4 cycles, amplitude 20%) for chromatin fragments approximately to the size of 250–500 bp. After centrifugation for 30 min, the supernatant of samples was normalized and the chromatin solution was subjected to O/N incubation at 4°C on a rotor with antibody (anti-POL II/anti-H3K14ac). Equilibrated Dynabeads (protein A and G) were added to each sample and incubated at 4°C for 3 h. Samples were washed with high salt buffer, low salt buffer, and LiCl buffer. The

samples were eluted in TE/SDS at 65°C for 15 min. Samples were kept for reverse crosslinking at 65°C. DNA extraction was carried out using PCI and the pellet obtained was resuspended in TE and followed by qPCR.

MNase Titration Assay

Briefly, 2 g leaf samples of *C. canadensis* from both the native (North America) and non-native (Kashmir Himalaya) ranges were collected and ground using liquid nitrogen. Nuclei extraction buffer (0.25 M sucrose, 60 mM KCl, 0.8% Triton x-100, 15 mM $MgCl_2$, 1 mM $CaCl_2$, 15 mM HEPES, 0.1 mM PMSF) was added to leaf samples for nucleus isolation. The isolated nucleus was then resuspended into MNase digestion buffer and incubated with different units of MNase (1U, 2U, 3.5U, and 5U) at 30°C for 10 min (Pajoro et al., 2018). The reaction was then stopped using a stop buffer (250 mM EDTA and 10% SDS). All the samples were then subjected to 3 µl RNase treatment and 1/2 h incubation. This was followed by proteinase K treatment followed by 1 h incubation. DNA was then extracted, purified, and resuspended in TE buffer. The pattern of digested chromatin was confirmed on a 2% agarose gel, while its quantity and purity analyzed using NanoDrop^{TM} (Thermo Fisher Scientific, Waltham, MA, United States). The digested chromatin was quantified using ImageJ software (NIH Java-based).

RESULTS

ABC Transporter Genes Show Reduced DNA Methylation in the Non-native Range of Kashmir Himalaya

Using methylated DNA immunoprecipitation (MeDIP) with a 5-methylcytidine antibody followed by quantitative real-time (qRT-PCR), we compared the DNA methylation level at the ABC transporter genes of *C. canadensis* in the native range of North America and non-native range of Kashmir Himalaya. MeDIP is an immunocapturing approach for unbiased detection of methylated DNA, wherein genomic DNA is sheared by sonication followed by immunoprecipitation using an antimethyl cytosine antibody (Fabio et al., 2009). The *C. canadensis* within the non-native range of Kashmir Himalaya showed significantly reduced DNA methylation at ABC transporter genes as compared to the native range of North America (p = 0.0072). The qRT-PCR results showed an IP/input value between 2.16 and 6.88 in samples from the native range of North America, while samples from the non-native range of Kashmir Himalaya showed significantly reduced IP/input values between 0.11 and 0.75 (**Figure 1**). Quite interestingly, the DNA methylation patterns of ABC transporter genes also varied across different populations within the native range of North America and the non-native range of Kashmir Himalaya. These results confirm that DNA methylation at ABC transporter genes could be one of the major factors regulating the expression of ABC transporter genes in the native range of North America and the non-native range of Kashmir Himalaya and that the reduced DNA methylation in the non-native range

of Kashmir Himalaya may lead to increased ABC gene expression and hence more adaptability.

ABC Transporter Genes Show Enriched Pol II Occupancy in the Non-native Range of Kashmir Himalaya

Using chromatin immunoprecipitation with RNA Pol II antibody followed by qRT-PCR, we looked at RNA polymerase-II occupancy at ABC transporter genes in the native (North America) and non-native (Kashmir Himalaya) leaf samples. Pol II ChIP detects RNA polymerase II within the coding region of genes using polymerase antibodies analyzed using PCR (Sandoval et al., 2004). As expected, we observed increased RNA Pol II occupancy at ABC transporter genes in the non-native range of Kashmir Himalaya as compared to the native range of North America (**Figure 2**). The qRT-PCR results showed Pol II occupancy value between 0.128 and 0.296 in leaf samples of the native range of North America, while the leaf samples from the non-native range of Kashmir Himalaya showed Pol II occupancy value between 0.8 and 1.206. Furthermore, like DNA methylation, RNA Pol-II binding at ABC transporter genes also showed a variation across the different sites both within the native range of North America and the non-native range of Kashmir Himalaya. Reduced DNA methylation and subsequent increased RNA Pol-II binding may bring about the transcriptional activation of ABC transporter genes in the non-native range of Kashmir Himalaya and hence their increased expression.

ABC Transporter Genes Show Loss of Nucleosomes in the Non-native Range of Kashmir Himalaya

In this study, we observed reduced acetylation levels of H3K14ac in the non-native range of Kashmir Himalaya as compared with the native range of North America, which could be due to changes in nucleosome occupancy (**Figure 3**). Chromatin immunoprecipitation analysis of histone H3 showed reduced nucleosome occupancy under non-native conditions. To get further insights into the changes in chromatin structure, we carried out micrococcal nuclease (MNase titrations) of chromatin from the native range of North America and the non-native range of Kashmir Himalaya. The MNase assay involves the digestion of chromatin using an enzyme MNase and understands the nucleosomal pattern by quantifying the protection of DNA from enzymatic digestion (Pass et al., 2017). Using the different concentrations of enzymes in an MNase assay, the non-native samples of Kashmir Himalaya showed more mononucleosomes as compared with the native ones of North America, indicating more open chromatin in the non-native *C. canadensis* within Kashmir Himalaya (**Figure 4**). This result suggests that the ABC locus in *C. canadensis* undergoes chromatin changes to provide phenotypic plasticity to the plant in the non-native range of Kashmir Himalaya. Collectively, reduced DNA methylation associated with increased Pol-II binding at the ABC transporter genes and open chromatin in *C. canadensis* within the non-native range of Kashmir Himalaya increases
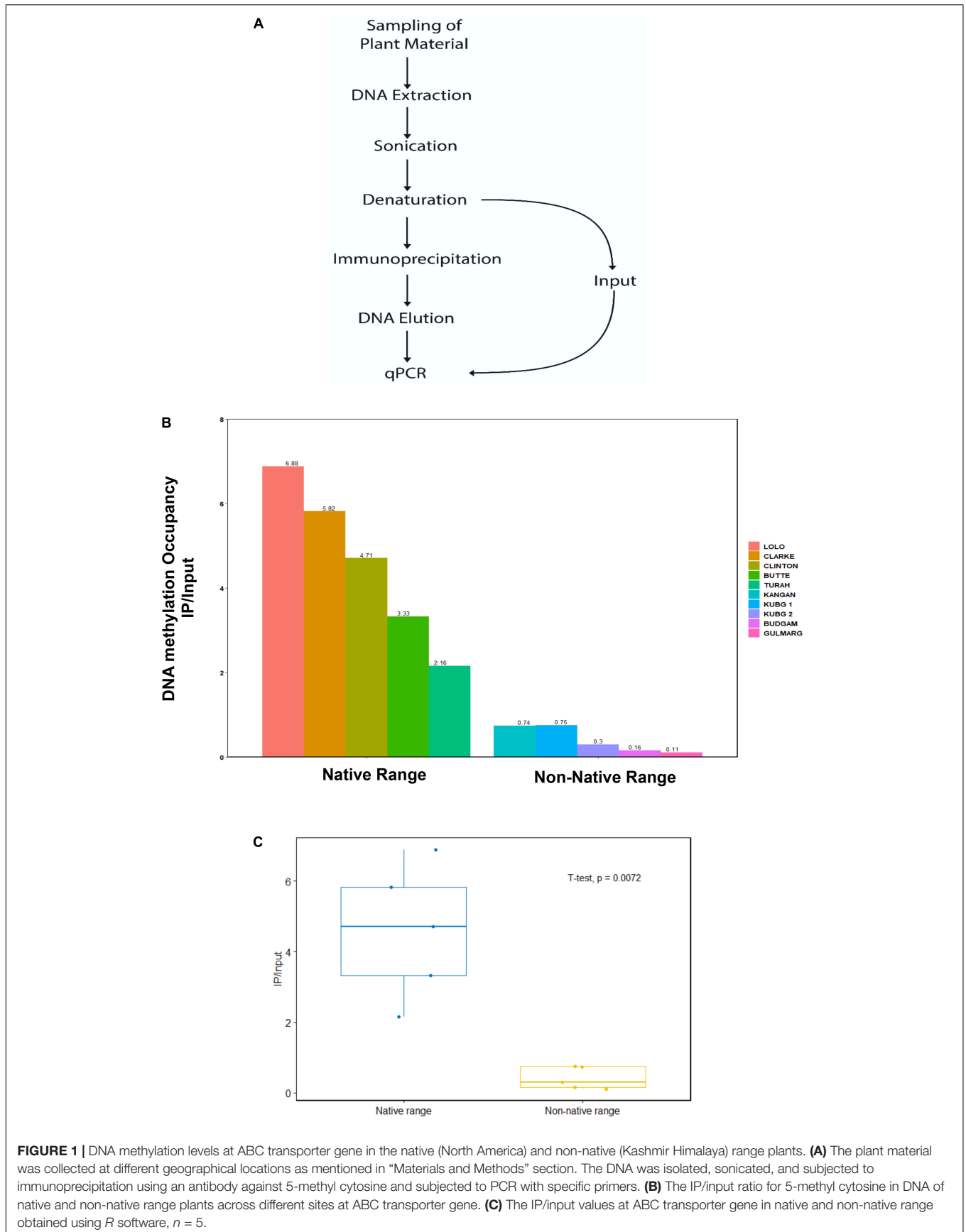
FIGURE 1 | DNA methylation levels at ABC transporter gene in the native (North America) and non-native (Kashmir Himalaya) range plants. **(A)** The plant material was collected at different geographical locations as mentioned in "Materials and Methods" section. The DNA was isolated, sonicated, and subjected to immunoprecipitation using an antibody against 5-methyl cytosine and subjected to PCR with specific primers. **(B)** The IP/input ratio for 5-methyl cytosine in DNA of native and non-native range plants across different sites at ABC transporter gene. **(C)** The IP/input values at ABC transporter gene in native and non-native range obtained using *R* software, *n* = 5.
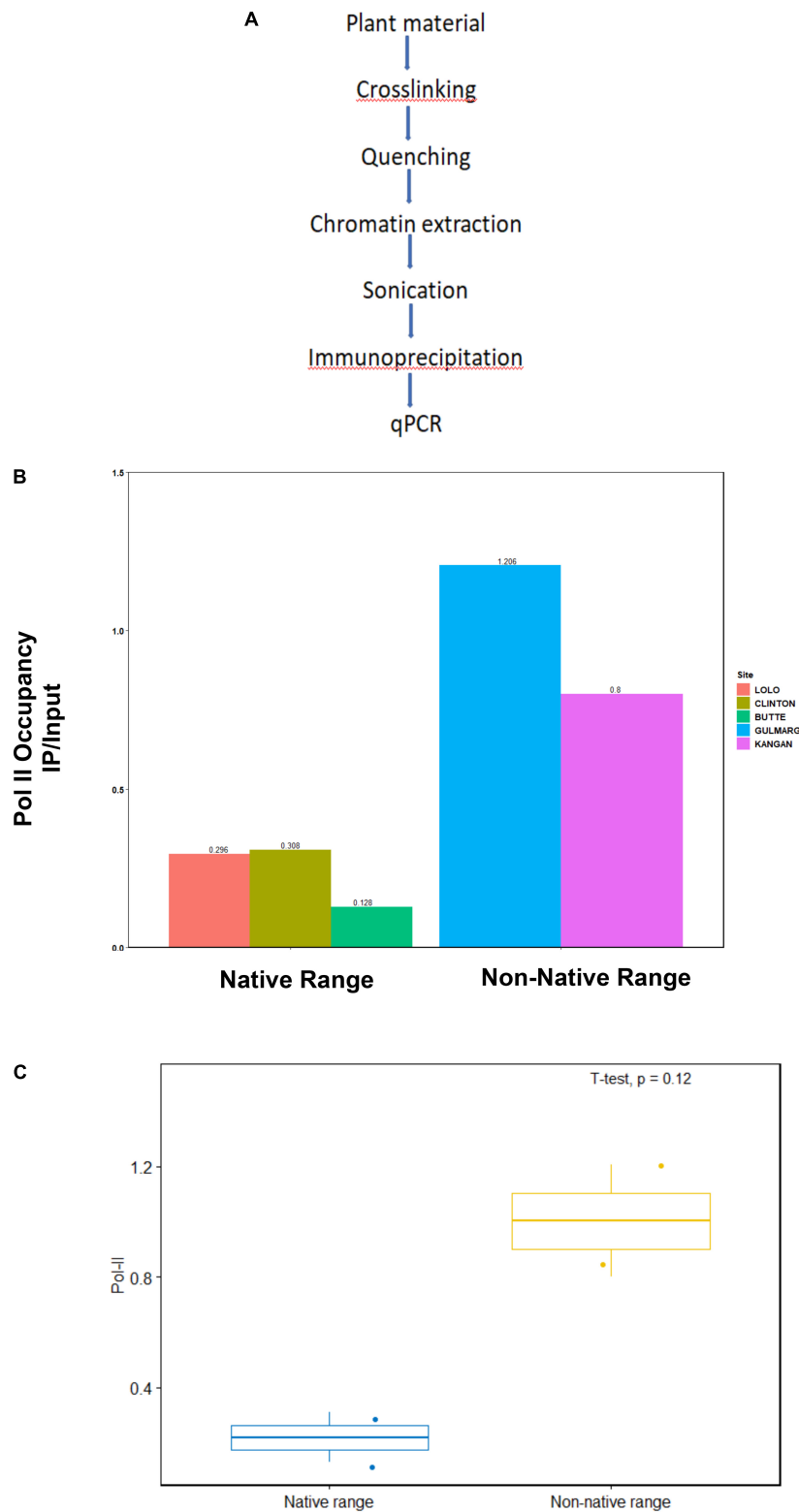
FIGURE 2 | Pol-II occupancy at ABC transporter genes in the native (North America) and non-native (Kashmir Himalaya) range plants. **(A)** The plant material was collected at different geographical locations as mentioned in "Materials and Methods" section. The DNA-protein interaction was cross-linked with a suitable cross-linking agent. Subsequently, the DNA was isolated, sonicated, and then subjected to immunoprecipitation using specific antibodies. **(B)** The bar graphs represent the RNA Pol-II occupancy at ABC transporter genes in native and non-native range plants. **(C)** RNA Pol-II occupancy at ABC transporter gene in the native and non-native range obtained using *R* software, *n* = 3.
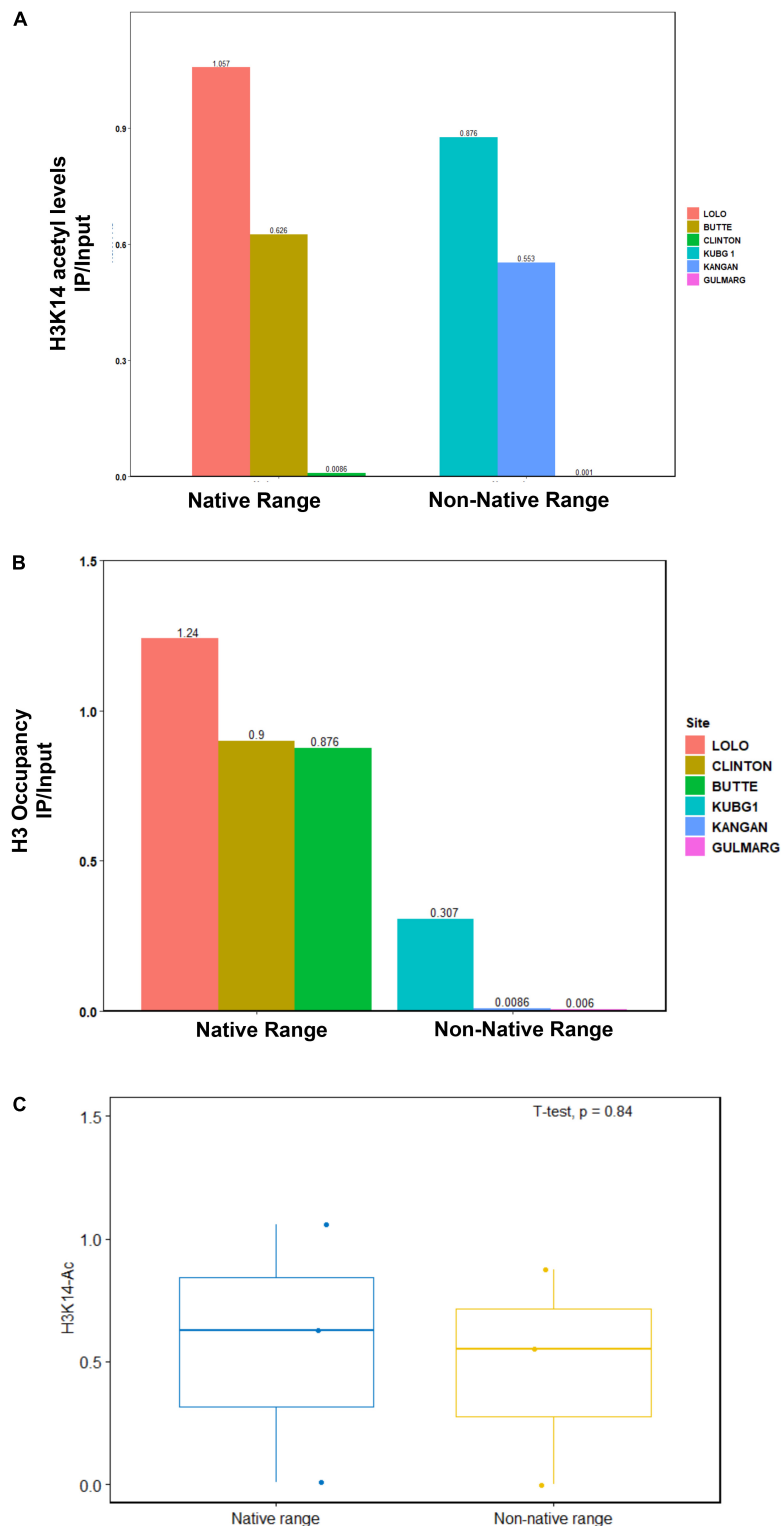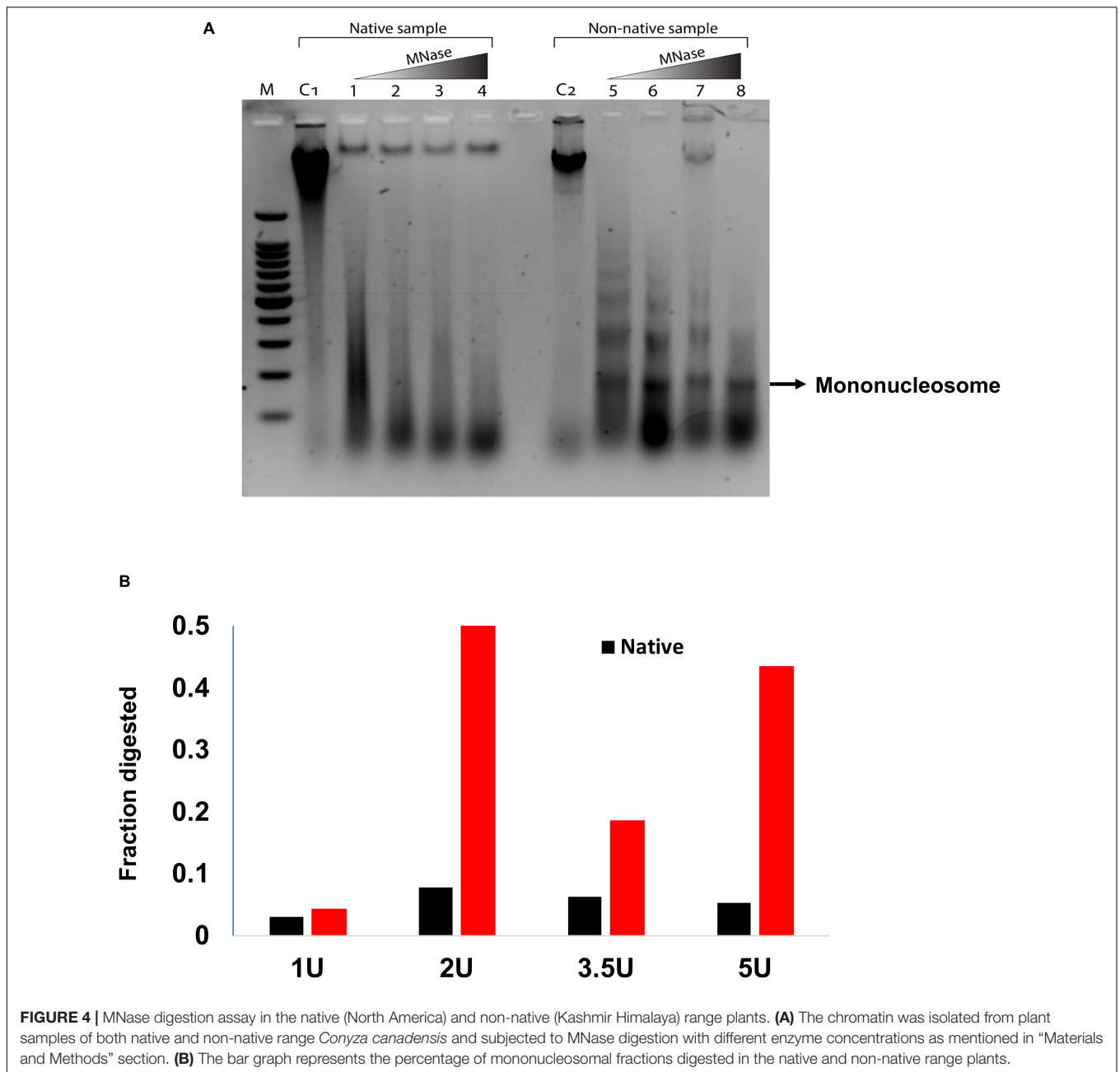
FIGURE 3 | H3 and H3K14 acetyl occupancy in the native (North America) and non-native (Kashmir Himalaya) range plants. The plant material was collected at different geographical locations as mentioned in "Materials and Methods" section. The DNA-protein interaction was cross-linked with a suitable cross-linking agent. Subsequently, the DNA was isolated, sonicated, and then subjected to immunoprecipitation using specific antibodies. **(A)** The bar graphs represent the IP/Input ratio of H3K14ac occupancy at ABC transporter genes in the native and non-native range plants. **(B)** The bar graphs represent the IP/Input ratio of H3 occupancy at ABC transporter genes in the native and non-native range plants. **(C)** H3K14 acetyl occupancy at ABC transporter gene in the native and non-native range obtained using *R* software, *n* = 3.
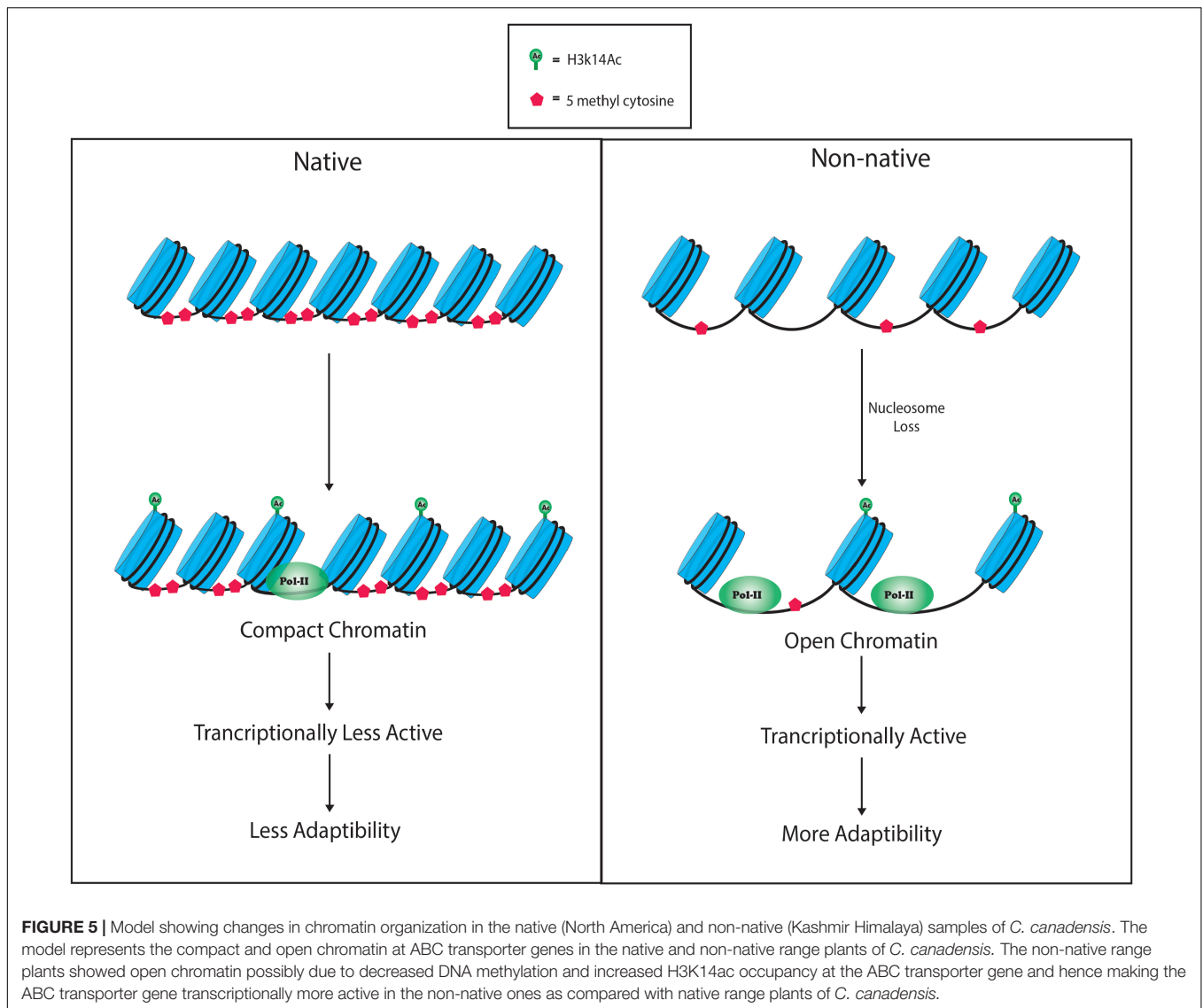
FIGURE 4 | MNase digestion assay in the native (North America) and non-native (Kashmir Himalaya) range plants. **(A)** The chromatin was isolated from plant samples of both native and non-native range *Conyza canadensis* and subjected to MNase digestion with different enzyme concentrations as mentioned in "Materials and Methods" section. **(B)** The bar graph represents the percentage of mononucleosomal fractions digested in the native and non-native range plants.

the transcriptional output that may increase the adaptability of these plants.

DISCUSSION

In this study, we observed a differential DNA methylation pattern at ABC transporter genes in the leaf samples of *C. canadensis* collected from its native range of North America and the non-native range of Kashmir Himalaya. The samples from the non-native range of Kashmir Himalaya showed a significantly lower DNA methylation level at ABC transporter genes as compared with the native samples of North America (**Figure 1**).

Furthermore, DNA methylation patterns also showed variation across different populations within the native and non-native ranges. Studies have shown the involvement of differential methylation patterns in the widespread distribution of species in the non-native ranges (Ardura et al., 2018; Hawes et al., 2019). These studies revealed that epigenetic changes vary in heterogeneous habitats and these changes are heritable. Studies in invasive plant species have shown a positive correlation between differential methylation status and phenotypic plasticity (Zhang et al., 2016; Banerjee et al., 2019). This shows that epigenetic diversity may contribute to the phenotypic plasticity in invasive species independent of genetic diversity. As a consequence of DNA methylation, chromatin compresses into

FIGURE 5 | Model showing changes in chromatin organization in the native (North America) and non-native (Kashmir Himalaya) samples of *C. canadensis*. The model represents the compact and open chromatin at ABC transporter genes in the native and non-native range plants of *C. canadensis*. The non-native range plants showed open chromatin possibly due to decreased DNA methylation and increased H3K14ac occupancy at the ABC transporter gene and hence making the ABC transporter gene transcriptionally more active in the non-native ones as compared with native range plants of *C. canadensis*.

heterochromatin at the transcription start site and hence reduces the gene activity and its transcription (Buitrago et al., 2021). The reduced DNA methylation observed in *C. canadensis* in the non-native range of Kashmir Himalaya might explain the increased expression of ABC transporter genes. Various studies have shown a direct link between the environmental condition and induction levels of key genes including ABC transporters and that these ABC transporters could be playing a role in the establishment and spread of *C. canadensis* in Kashmir Himalaya (Tani et al., 2016). A link has been deciphered between DNA demethylation and invasion during the stage of expansion (Ardura et al., 2017). This may happen because of gene activation and reactivation of transposable elements redefining gene expression. As a result, demethylation may confer varying phenotypic plasticity and fitness. Plants have at least three different DNA methyltransferases, also called cytosine methyltransferases, which differ in structure and function and methylates DNA at cytosine (m5C) and adenine

(m6A) (Finnegan and Kovac, 2000; Vanyushin, 2006). These various classes of DNMTS within the plant system, unlike the mammalian system, show differential methyltransferase activity and context-dependent effect on cytosine base (Finnegan and Kovac, 2000). Given that we observed the differential DNA methylation patterns in native (North America) and non-native (Kashmir Himalaya) *C. canadensis*, it will be an interesting area of research to profile the *C. canadensis* in other native and non-native ranges for DNA methylation and differential DNA methylation patterns. Since, we hypothesize that DNA associated Pol-II elongation may increase the RNA Pol-II binding at ABC transporter genes (Jonkers and Lis, 2015), we observed increased RNA Pol-II binding in non-native (Kashmir Himalaya) range *C. canadensis* as compared with native (North America) samples (**Figure 2**). This result is in line with the fact that reduced DNA methylation is mostly associated with increased RNA Pol-II binding and hence results in increased gene expression (Bender, 2004; Takeshima et al., 2009). Furthermore, we observed the

loss of nucleosome at the ABC gene transporters in non-native samples of Kashmir Himalaya as indicated by comparative ChIP analysis of H3K14ac and H3 (**Figure 3**). This loss of nucleosomes is an indication of open chromatin in the non-native range of Kashmir Himalaya. Therefore, we also compared the chromatin dynamics in *C. canadensis* within its native (North America) and non-native range (Kashmir Himalaya) using MNase assay. In this study, nucleosomal occupancy and positioning have a critical impact on the expression and regulation of genes (Zhang et al., 2015). The MNase assay performed on the leaf samples of *C. canadensis* from native (North America) and non-native (Kashmir Himalaya) range revealed an interesting phenomenon of chromatin changes in non-native range plants of Kashmir Himalaya. The non-native plants of Kashmir Himalaya showed an increased mononucleosomal fraction as compared with native ones of North America (**Figure 4**). The increased mononucleosomal fraction is an indication of open and more accessible chromatin in non-native plants of Kashmir Himalaya that may modulate gene expression of these plants in the non-native range of Kashmir Himalaya (Kenchanmane Raju, 2020).

Taken collectively, our study has identified the differential DNA methylation patterns at ABC transporter genes in the native (North America) and non-native (Kashmir Himalaya) samples of *C. canadensis*. Non-native leaf samples of Kashmir Himalaya showed reduced DNA methylation at these genes, which was further associated with enhanced Pol-II binding. The combinatorial effect of reduced DNA methylation and increased RNA Pol-II binding along with open chromatin may enhance the transcriptional activity of ABC transporter genes and finally may increase the plant growth in different environmental conditions and may be one of the possible mechanisms through which plant becomes invasive in its non-native range of Kashmir Himalaya (**Figure 5**). Although our study deals with the chromatin dynamics at the ABC transporter genes, understanding the downstream events could be an exciting area of research.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

AS carried out the experiments. AS, ZR, and MA designed the experiments, interpreted the results, and wrote the manuscript. All authors edited and approved it.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2021.781498/full#supplementary-material

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