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Plant Genetic Bases Associated With Microbiota Descriptors Shed Light Into a Novel Holobiont Generalist Genes Theory

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ABSTRACT

Plants as animals are associated with a cortege of microbes influencing their health, fitness and evolution. Scientists refer to all living organisms as holobionts, complex genetic units that coevolve simultaneously. This is what has been recently proposed as the hologenome theory. This exciting theory has important implications on animal and plant health; however, it still needs consistent proof to be validated. Indeed, holobionts are still poorly studied in their natural habitats where coevolution processes occur. Compared to animals, wild plant populations are an excellent model to explore the hologenome theory. These sessile holobionts have coevolved with their microbiota for decades, and natural selection and adaptive processes acting on wild plants are likely to regulate the plant–microbe interactions. Here, we conducted a microbiota survey, plant genome sequencing and genome–environmental analysis (GEA) of 26 natural populations of the plant species *Brassica rapa*. We collected plants over two seasons in Italy and France and analysed the root and rhizosphere microbiota. When conducting GEA, we evidenced neat peaks of association correlating with both fungal and bacterial microbiota. Surprisingly, we found 13 common genes between fungal and bacterial diversity descriptors that we referred to under the name of holobiont generalist genes (HGGs).

1 | Introduction

With no exceptions, all living organisms are associated with microorganisms (viruses, prokaryotes, fungi, protists) that are commonly referred to as the host–microbiome. Evolutionary Scientists coined the terminology of holobiont for describing hosts and their associated microbes as a unique entity on which natural selection operates. Therefore, in the last decade, there is a significant shift in the way that evolution is considered by evolutionary scientists; indeed, animals and plants are no longer a centric and sole protagonist of evolution but rather an entity much more complex and universal (Bordenstein and Theis 2015; O'Brien et al. 2019; Stencel and Wloch-Salamon 2018). More largely, the evolutionary core is not the individual genome anymore but what is called the hologenome, that is, the genome of the host and of all associated microscopic organisms. Accepting the hologenome as a valid

biological unit is equivalent to assuming that selection directly acts on holobionts as they are unique entities in constant coevolution (Zilber-Rosenberg and Rosenberg 2008). But how has this exciting and promising theory of evolution been studied so far? In animals, particularly humans, the microbiota's role in health and disease has been extensively studied, providing strong evidence of its influence on digestion, immunity, metabolism and even mental health. For example, studies show that alterations in gut microbiota composition contribute to diseases such as obesity, diabetes, inflammatory bowel disease (IBD) and neurological disorders like autism and depression (Turnbaugh et al. 2006; Gilbert et al. 2018). However, despite the increasing research in this field, conclusive, tangible evidence supporting the hologenome theory as a universally applicable evolutionary framework remains elusive. The main limiting factor is the concern that studies often show correlations between microbiota composition and host traits, but proving causal evolutionary changes is challenging (Spor et al. 2011). In addition, even if genetic adaptation in hosts is well documented, the evidence that microbiota-induced changes persist across multiple generations is still limited.

Compared to animals, sessile organisms, such as plants, are an exceptional resource to study the hologenome concept as they are forced to adapt to the fluctuating environment where they evolve. Therefore, they strongly coevolve with soil microbes living in their ecological habitats. It has been proposed, but not entirely proved, that plants can modulate their microbiomes to dynamically adjust to their habitats (Vandenkoornhuyse et al. 2015). If confirmed, this observation can hugely change agriculture as we could breed crop varieties specifically selecting for beneficial microbes. As the host plant genome slowly evolves and the microbiota quickly change (Madhusoodanan 2019), the use of the holobiont concept in agriculture can improve crop systems in a short-time scale. As reviewed in Spooren et al. (2024) plants selectively shape their microbiomes through root exudates, creating microenvironments that support beneficial microbes. Plant immunity and genetic traits govern microbiome assembly, offering adaptive advantages to the holobiont (Hacquard et al. 2017). These advantages are evident in disease-suppressive soils, where specific microbes accumulate to inhibit pathogens after an initial disease outbreak (Jayaraman et al. 2021). As proposed in Spooren et al. (2024) in response to pathogen attacks, there is a 'cry for help' by recruiting protective microbes, thereby establishing a soilborne legacy that safeguards subsequent plant generations. Integrating microbiomes into farming practices offers promising avenues for sustainable agriculture. By leveraging beneficial microorganisms, farmers can enhance crop growth, improve soil health and manage pests more effectively. Microbial-based biopesticides, have shown increased potency against insect pests, offering a more targeted and eco-friendly alternative to chemical pesticides (Quadri et al. 2020). Additionally, the application of plant-growth-promoting microbes can enhance nutrient uptake and plant resilience to environmental stresses. Emerging strategies, including the development of Synthetic Microbial Communities (SynComs) and the use of organic soil amendments as prebiotics, will promote the establishment of beneficial plant–microbiome associations (Batista and Singh 2021).

However, before using microbes in real farming situations, we still have several unanswered questions to address. For instance, how plant holobionts can be studied and how information on the coevolution between hosts and microbiomes can improve agriculture? In the current genomic era, two main approaches can be adopted to improve knowledge on holobionts. Firstly, experimental evolution (Ramiro et al. 2021; Batstone et al. 2020) coupled with genomics can help identifying rapid evolutionary changes in both host and microbiome genomes. Experimental evolution is definitely a powerful tool, still underestimated and underused; however, it only allows the study of holobionts on a short ecological and evolutionary time-frame. Alternatively, genome–environmental analysis (GEA) allows to capture an entire picture of natural evolutionary processes in holobionts. Indeed, GEA can identify significant associations between the host genetic polymorphisms and environmental variables such as microbiome descriptors. As mentioned in Fitzpatrick et al. (2020) 'The next stage in plant microbiome research will require the integration of ecological and reductionist approaches to establish a general understanding of the assembly and function in both natural and managed environments'. In light of this, GEA is an alternative to identify host genes involved in adaptive processes of the holobiont unit. Recently, studies showed the power of GEA to identify plant loci associated with biotic communities. This has been demonstrated both in the non-model plant species *Brassica incana* and its associated pollinator communities (Frachon et al. 2023), and in the model plant species *Arabidopsis thaliana* and its plant–plant (Frachon et al. 2019) and plant–microbiome (Roux et al. 2023) interactions. The latter study is the only describing adaptive loci associated with bacterial descriptors in natural plant populations. So far, GEA studies on plant–microbiome models are rare, especially on non-model plant species. *Brassica* species are a great genetic resource as wild *Brassica* populations—closely related to crops—still exist in a wide range of habitats. This fascinating genetic material is still poorly studied and can open the avenue into the elucidation of holobionts' evolution (Falentin et al. 2024).

Here we focus on the genetic architecture of the plant–microbiome interaction in wild *Brassica rapa* populations, a non-model plant and one of the two diploid progenitors of oilseed rape (*Brassica napus*). Wild *B. rapa* populations are closely related to various cultivated forms including turnips (*B. rapa* subsp. *rapa*). We ecologically and genetically characterised 26 wild *B. rapa* populations and produced information on the fungal and bacterial microbiome over two seasons and plant compartments (root and rhizosphere). We adopted a GEA approach and found that genetic bases controlling microbiota diversity and composition are shared between fungal and bacterial communities. Here we refer to plant genes shared among microbiota fungal and bacterial descriptors as 'holobiont generalist genes (HGGs)' since they regulate holobionts at different kingdoms levels.

2 | Results

2.1 | Genetic and Ecological Description of *B. rapa* Natural Populations Showed Highly Genetically Differentiated Populations in the South of Italy

To explore the long-term coevolution between plants and their associated microbes, we chose to work on wild *B. rapa* populations as, compared to crops, they are locally adapted to their habitats and microbiomes. Twenty-six populations were collected during 2020 in both the south of Italy—Sicily, an Italian

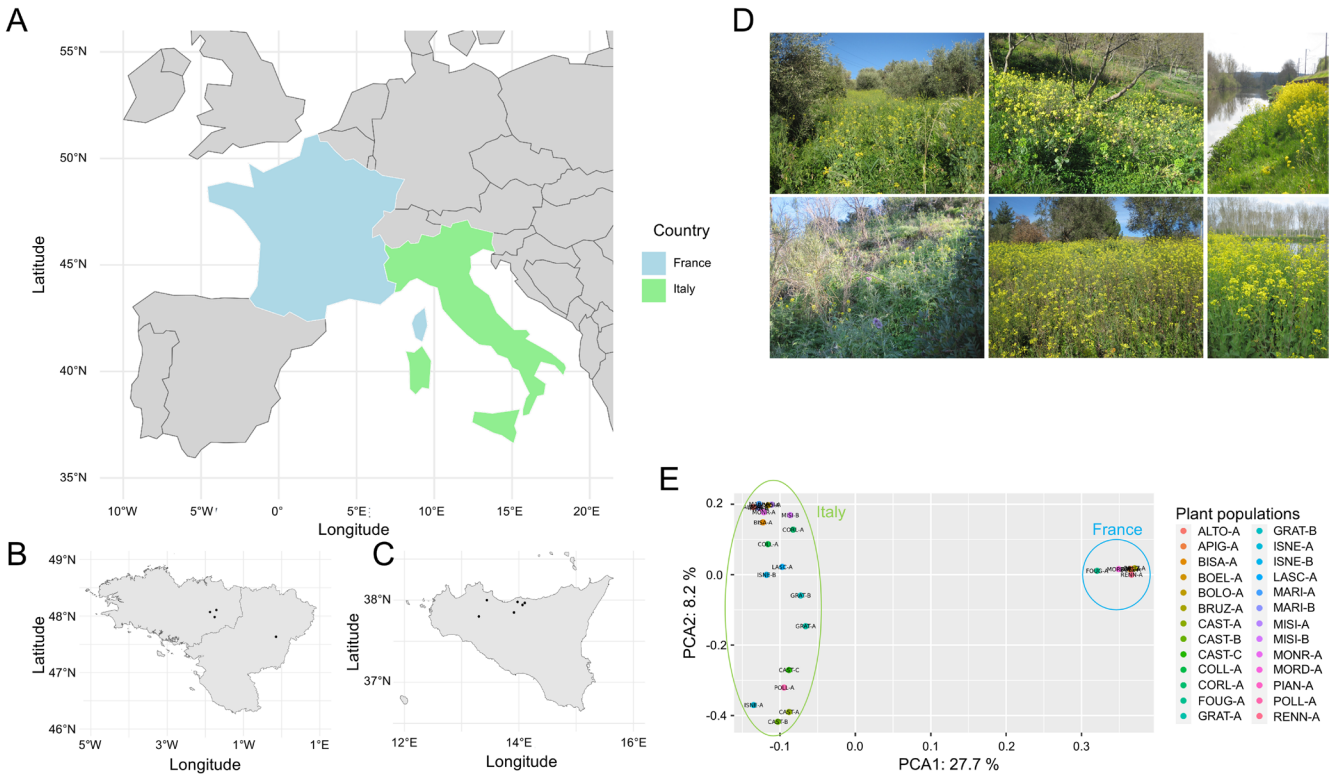


FIGURE 1 | Ecological description of *Brassica rapa* natural *populations* collected in this study. (A) Map of western Europe highlighting France (in light blue) and Italy (green). (B) Map of Brittany and Pays de la Loire (French departments); dark plots represent the six French *B. rapa* populations. (C) Map of Sicily (Italy island); dark plots represent the 20 Italian *B. rapa* populations. (D) Examples of the ecological habitats where *B. rapa* populations were collected in both Italy (two upper-left and two bottom-left panels) and France (upper-right and bottom-right panels). (E) Principal component analysis (PCoA) built on the SNP matrix of the 26 plant populations; each population is represented with a different coloured dot. Percentage of the explained variance for the two first principal components (PCs) (*x-axis* indicates the first principal component, and *y-axis* indicates the second principal component) is reported. The Italian populations are grouped under the green panel; otherwise, French populations are grouped under the blue panel.

island (20 populations)—and the north of France—Brittany and Pays de la Loire (6 populations)—(Figure 1A–C, Data S1). Plant habitats were selected to maximise the ecological variability (Figure 1D) in terms of climatic, soil, land use, altitude and plant community's diversity (Data S1). Plant collection is part of a larger H2020 Prima project 'BrasExplor' aiming at characterising the genetic diversity of *Brassica* species in the Mediterranean basin (Falentin et al. 2024). During fall 2020, we collected 30 plants for each of the 26 populations and sequenced each of them using a PoolSeq approach. We obtained 86,989,331.69 (average), 88,727,527.00 (median), 4,937,501.74 (SD) reads per library (Data S2). Raw data were filtered based on sequences' quality. After trimming, we obtained 86,964,161.23 (mean), 88,677,517.00 (median), 4,940,310.81 (SD) reads. Prior to genome mapping, we sequenced the reference genome of *Brassica napus* RCC-S0 (Supporting Information Text), a synthetic line composed of *B. rapa* C1.3 A-subgenome. We adopted this method as *B. rapa* C1.3 is a variety genetically close to the wild *B. rapa* populations (Supporting Information Text, Data S3, Figure S1). Wild *B. rapa* population genomes were mapped on the *B. rapa* C1.3 genome. After mapping and SNP calling, we obtained a matrix of 12,518,450 SNPs that were trimmed. The final data matrix was composed of 1,138,924 SNPs.

The genomic PCoA on the SNPs matrix of the 26 populations (Figure 1E) showed that principal component 1 partly explains the variation among the countries, and principal component 2 explains the variation within each country (mostly for the Italian populations). We observed that the Italian populations were more scattered in the genomic space compared to those collected in France (Figure 1E). This is probably related to the ecological diversification of the habitats where plants were collected. Indeed, populations from Italy were located in highly diversified habitats ranging from abandoned fields to olive orchards (Figure 1D, Data S1) and showed a larger diversity in terms of plant communities (Figure S2, Data S1). On the other hand, French populations were all collected closer to rivers (Figure 1D) and habitat characterisation showed, as expected, that plant communities were extremely different from those observed in Italy (Figure S2, Data S1). Nevertheless, other factors, for example, the *B. rapa* populations demography history, might have influenced the genomic differentiations of the Sicilian populations.

2.2 | *Brassica rapa* Microbiota Descriptors Are Influenced by Plant Compartment, Season and Plant Genotype

To study the interactions within the *B. rapa* populations and their associated microbiomes, we conducted an in situ microbiome survey over two seasons: (i) spring (SP), corresponding

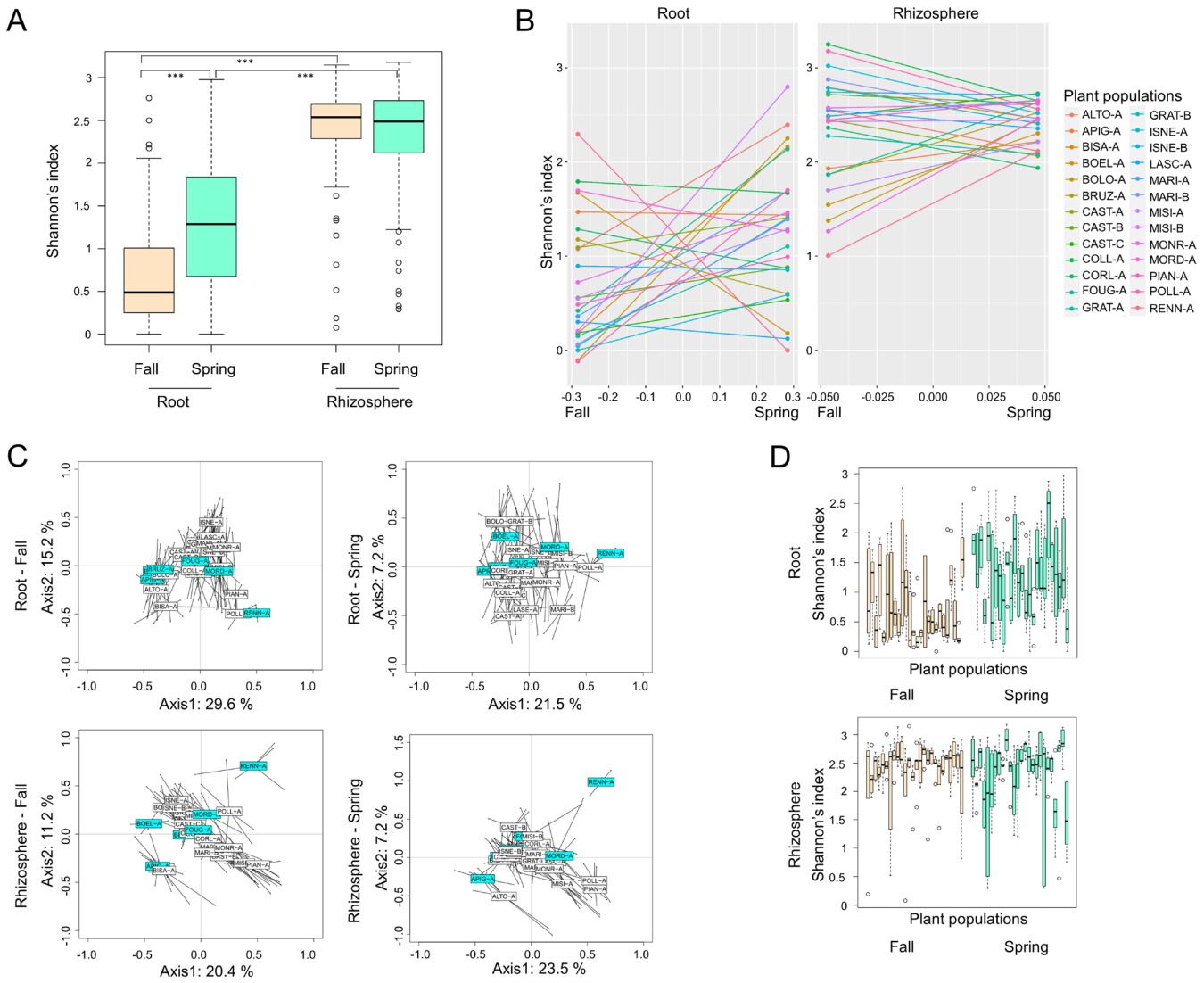


FIGURE 2 | Fungal microbiota descriptor analysis. (A) Box plots on Shannon's index (*y*-axis) showing the variation of fungal α-diversity within plant compartments (root and rhizosphere, *x*-axis) and seasons: Fall represented by pale brown and spring represented by mint green. Significant differences are indicated, with '***' representing *p*-values ≥ 106. (B) Finlay Wilkinson's regression for Shannon's index (*y*-axis) of the 26 *Brassica rapa* plant populations over two seasons (fall and spring, *y*-axis) and for each plant compartment (left: root, right: rhizosphere). Each colour represents a different population. French and Italian populations showed no pattern. (C) Transformation-based-redundancy analysis (tbRDA) plot representing the fungal composition variation within the 26 *B. rapa* populations run on four datasets: Top-left: Root-fall, top-right: Root-spring, bottom-left: Rhizosphere-fall, bottom-right: Rhizosphere-spring. French populations are represented by blue boxes. (D) Box plots illustrating the seasonal effect on Shannon's index (*y*-axis) estimated on root (top panel) and rhizosphere (bottom panel) compartments over two seasons: Fall represented by pale brown and spring represented by mint green. Each plot represents Shannon's index of a specific plant population (*x*-axis).

to February 2020 in Italy, and May 2020 in France—plants were at the flowering stage—and (ii) fall (FA), corresponding to December 2020 in Italy, and January 2021 in France; all plant populations were at the 4-leaves stage in fall. For microbiome description, we collected 6 plants per population and we directly separated in the field root (R) and rhizosphere (RH) compartments. We amplified a portion of the ITS1 and *rpoB* housekeeping gene marker (Ogier et al. 2019) for fungal and bacterial characterisation communities respectively. For bacterial communities, root samples were removed because of their poor raw data quality. Concerning the fungal communities, we observed a strong plant compartment effect on diversity α-descriptors but not on the fungal composition (Figure 2A, Figure S3, Table S1). Not surprisingly, fungal diversity was higher in the rhizosphere than in the root compartment. We also evidenced a significant effect of the season variable on fungal diversity but not on composition. This effect was significant in the root compartment but not in the rhizosphere (Figure 2A,B, Table S2). As both season and plant compartment strongly influenced fungal communities, analyses were run on four datasets to test whether plant genotype has an impact on microbiome descriptors. The four datasets are named as: root in fall (R-FA), root in spring (R-SP), rhizosphere in fall (RH-FA) and rhizosphere in spring (RH-SP). By running a transformation-based Canonical Redundancy Analysis (tb-RDA) and linear-mixed models on the PCoA axis, we identified a strong plant population effect on the fungal composition on both R and RH compartments and over the

two seasons (Figure 2C, Tables S3, S4). The plant population variable also strongly influenced α-diversity descriptors (Figure 2D, Figure S4, Table S4).

For bacterial communities we only reported data on the rhizosphere microbiome as a large majority of root samples were characterised by less than 1000 reads after sequencing. We therefore removed the root dataset for the poor quality of the obtained sequences. As for the fungal microbiome, we did not observe a seasonal effect on rhizosphere samples (Figure 3A,B, Figure S5, Table S5). On the other hand, we observed a significant effect of the plant population variable on both bacterial community composition (Figure 3C, Table S4, Table S6) and α-diversity (Figure 3D, Table S4). Globally, for both fungal and bacterial communities we observed a significant plant population effect on both microbiome α-diversity descriptors and composition. We therefore estimated the broad-sense heritability (H^2) on microbiota descriptors (Shannon's index, species richness and the two first axis of the PCoA) and observed significantly high values of H^2 for most of the fungal and bacterial descriptors (Table S7). These results claim a crucial role of plant genetic diversity in modulating both fungal and bacterial communities. We then decided to couple information on plant population genetic diversity with microbiome descriptor analysis to better define the genetic architecture of the *B. rapa* holobiont.

2.3 | Genome–Environmental Analysis Showed a Highly Diversified Plant Genetic Architecture Within Plant Compartments and Seasons as Association With Plant Resistance Genes

To identify the genetic bases associated with both fungal and bacterial community descriptors, we adopted a GEA approach by combining a Bayesian hierarchical model (BHM) that decreases the rate of false genotype-ecology positive associations (Gautier 2015) with a Local Score (LS) approach allowing the detection of QTLs with small effects (Bonhomme et al. 2019). The BHM model integrated in the BayPass software (Gautier 2015) used in our study is suitable as it allows control for population structure by estimating a covariance matrix on the plant SNPs matrix. The covariance structure among the population allele frequencies resulted from the shared history of the populations under study (Gautier 2015). best-linear-unbiased-predictions (BLUPs) were estimated on α-microbiota descriptors, community composition descriptors and on the relative abundance of the most prevalent OTUs (Data S5–S10) by controlling for the *population* and the *replicates* factors (as indicated in the Material and Methods Section 4). The plant trimmed SNPs matrix was then integrated in the BHM model estimating the covariance matrix minimising for population structure among the Italian and French populations. BLUPs were integrated as covariables to estimate the associated SNPs. After running the LS approach, we selected only QTLs that had a significant local score value (Lindley score), and we retrieved genes falling in these QTLs. For both fungal and bacterial communities, we found that the genetic architecture is highly variable among seasons and plant compartments, as few genes were found to be shared within root and rhizosphere samples, and within fall and spring when analysing fungal and bacterial communities separately (Figures S6, S7).

Regarding fungal microbial descriptors, we found neat peaks of association (i.e., carrying high LS) falling in genome regions corresponding to annotated *B. rapa* genes (Data S11, S12). As very little information is available on *B. rapa* gene function, we searched their orthologs in the closely related plant model species *A. thaliana*. We then retrieved the known functions on TAIR website https://www.arabidopsis.org/. Among genes falling into the most significant association peaks, we found the A01p01520.1_BnaRCC gene associated with the relative abundance of the fungal OTU000080 that corresponds to the well-known plant-protecting fungal species *Trichoderma* sp. (Data S11, S12). We also found that the A02p35100.1_BnaRCC gene was associated with the fungal PCoA axis 4 (Data S11, S12). Both genes correspond to members of the cytochrome P450 (CYP) in *A. thaliana*. CYP is an ancient superfamily of enzymes involved in multiple catalytic pathways and identified in all domains of living organisms (Baldwin et al. 2009). We evidenced a neat peak of association on chromosome 7 to be associated with the OTU000048 belonging to the fungal species *Preussia flanaganii*. We retrieved the gene A07p21630.1_BnaRCC (Supporting Information Data S11, S12) corresponding in *A. thaliana* to the Basic Helix–Loop–Helix protein 100 (bHLH100), which is one of the largest transcription factor gene families in *Arabidopsis* and which is involved in the resistance to abiotic stresses (Hao et al. 2021). We identified several SNPs within genes known to be related to plant resistance pathways. For instance, A01p08480.1_BnaRCC is associated with the relative abundance of the fungal OTU000050 *Fusicolla* sp. (Supporting Information S11, S12). Its *A. thaliana* orthologous gene is the IBI1 receptor that is responsible for BABA-induced resistance. Also, we found A09p17430.1_BnaRCC to be associated with the relative abundance of the fungal OTU000079 belonging to the *Vishniacozyma* (Supporting Information S11, S12) species that are known to induce resistance to fungal pathogens. Its *A. thaliana* ortholog corresponds to the BURNOUT1 gene that is related to several plant stress responses.

We also found several neat peaks associated with bacterial descriptors (Supporting Information S11). For example, we identify a significant peak of association in the A07p36280.1_BnaRCC gene (encoding a deoxyribonucleoside kinase: ATDNK) to be associated with bacterial species richness (Supporting Information S11, S12). Interestingly, we found a significant peak on chromosome 9 to be associated with the bacterial species *Bacillus simplex* (Supporting Information S11, S12) described to be a biocontrol agent against *Fusarium* fungal pathogens and other plant pathogens, as most *Bacillus* species (Al-Sman et al. 2019; Shafi et al. 2017). The peak fell in the A09p41890.1_BnaRCC gene, whose *A. thaliana* ortholog corresponds to the TIP41-like protein that was shown to be involved in the plant defence against bacterial pathogens (Punzo et al. 2018).

2.4 | Common Genetic Bases Associated With Both Fungal and Bacterial Communities Drive Into a Novel Theory of Holobiont Generalist Genes (HGGs)

One of the aims of our study was to explore whether genetic bases associated with microbiome descriptors are shared among the bacterial and fungal communities. We therefore intersect genes found to be associated with fungal and

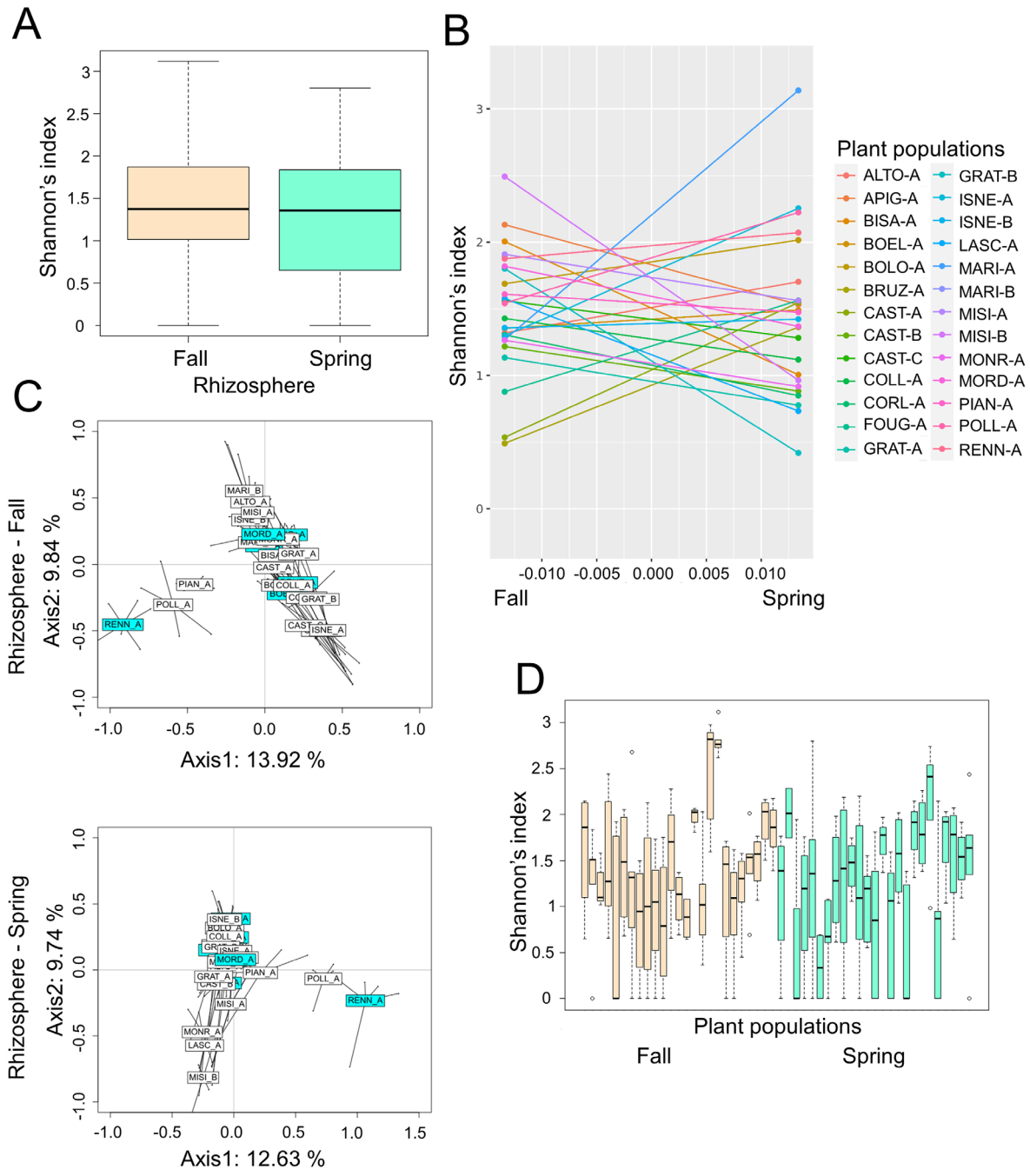
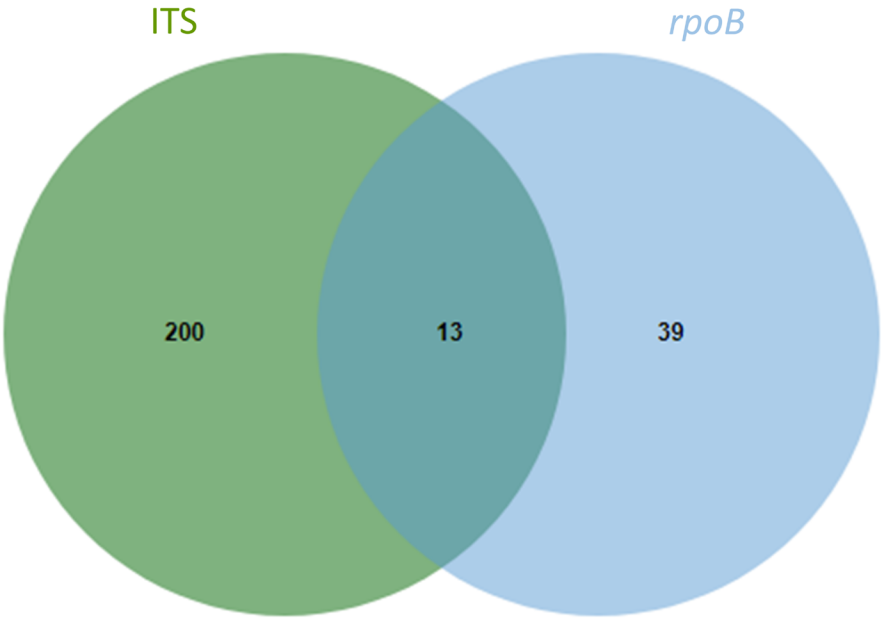


FIGURE 3 | Bacterial microbiota descriptor analysis. (A) Box plots on Shannon's index (*y*-axis) showing the variation of bacterial α-diversity within two seasons: Fall represented by pale brown and spring represented by mint green. (B) Finlay Wilkinson's regression for Shannon's index (*y*-axis) of the 26 *Brassica rapa* plant populations over two seasons (fall and spring, *y*-axis) for the root compartement. Each colour represents a different population. French and Italian populations showed no pattern. (C) Transformation-based-redundancy analysis (tbRDA) representing the bacterial composition variation within the 26 *B. rapa* populations run on two datasets: Fall (top panel) and spring (bottom panel). French populations are represented by blue boxes. (D) Box plots illustrating the seasonal effect on Shannon's index (*y*-axis) estimated over two seasons: Fall represented by pale brown and spring represented by mint green. Each plot represents Shannon's index of a specific plant population (x-axis).

bacterial descriptors. Surprisingly, when merging all compartments and seasons, we found that 13 genes were shared among microbiome descriptors (Figure 4). For instance, we found a neat peak within the A04p30810.1_BnaRCC gene associated with both the relative abundance of the fungal species *Cystofilobasidium macerans* and the bacterial species richness (Figure 5). This gene was also associated with the relative abundance of *Curvularia spicifera* and the ITS PCoA axis4 (Data S11). The A04p30810.1_BnaRCC Arabidopsis ortholog, the AT2G43610 gene, belongs to a chitinase family protein that is involved in root *Arabidopsis* morphogenesis (Kwon et al. 2015). A neat peak of association was found within the A06p13350.1_BnaRCC gene associated with both the relative abundance of the fungal species *Pseudeurotium* sp. and the relative abundance of the bacterium *Arthrobacter* sp. (Figure 5). The *A. thaliana* homologous AT1G18160 belongs

A



B

Brassica rapa gene name and *A. thaliana* homologous	N°of shared microbiome descriptors
A04p30810.1_BnaRCC: Chitinase family protein	**5**
A06p01040.1_BnaRCC: O-fucosyltransferase family protein	**4**
A09p50420.1_BnaRCC: Casein Lytic Proteinase	**4**
A09p41890.1_BnaRCC: TIP41-like protein	**3**
A07p36280.1_BnaRCC: ATDNK Deoxyribonucleoside kinase DNK	2
A05p25360.1_BnaRCC: Arabidopsis NPK1-activating kinesis 1	2
A05p25370.1_BnaRCC: NA	2
A05p25380.1_BnaRCC: NA	2
A06p13350.1_BnaRCC: M3KDELTA7	2
A06p17300.1_BnaRCC: Plant invertase	**2**
A06p17310.1_BnaRCC: Golgi S-adenosyl methione transporter 3	2
A07p24690.1_BnaRCC: HPPR2 Hudroxyphenilpyruvate reductase 2	**2**
A05p28030.1_BnaRCC: DNA-binding transcription factor 2	2

FIGURE 4 | Analysis on the holobiont generalist genes (HGGs) shared between fungal and bacterial descriptors. (A) Venn diagram showing the 13 genes shared among fungal (in green) and bacterial (in blue) genes that were found to be significantly associated with microbiota descriptors. (B) Function of the 13 shared genes inferred from *Arabidopsis thaliana*. Genes in bold are those associated with significant XtX values (i.e., genes under natural selection). NA = not attributed, indicates that gene functions were not found for the *A. thaliana* homologous.

to the M3KDELTA7 family that is involved in complex osmotic stress and abscisic acid (ABA) signal transduction network (Takahashi et al. 2020). Interestingly, we observed that the A09p41890.1_BnaRCC gene, which is the homologous of a TIP41-like, was shared among the fungal *Preussia flanaganii* relative abundance and bacterial species richness and *Bacillus simplex* abundance (Figure 4, Data S11).

Intriguingly, on chromosome 5, three successive genes A05p25360.1_BnaRCC, A05p25370.1_BnaRCC and A05p25380.1_BnaRCC were all associated with the bacterial composition (PCoA axis 3) and with the relative abundance of the fungal species *Cladosporium delicatulum* (Figure 4, Figure S8, Data S11). We identified that the Arabidopsis homologous gene of A05p25360.1_BnaRCC belongs to an NPK1-activating kinesin-like protein. However, no gene function was found for both A05p25370.1_BnaRCC and A05p25380.1_BnaRCC to evaluate whether the three genes participate in the same metabolic pathways. As gene regulation acts in cis (within one protein), structural interactions between proximally located genes generate genetic interactions of what is called causal genes (Diss and Lehner 2018). These causal genes are likely to be detected physically close in GEA, as we observed.

In our study, we referred to alleles found to be common between fungal and bacterial descriptors as HGGs. We found that HGG are likely to regulate species richness, composition and abundance of the host–microbiome, and therefore they are expected to play several roles in modulating plant–microbiomes over holobionts evolution. Additionally, our results suggest that HGG not only act at a global microbiome level, but they can also physically interact within themselves.

2.5 | Holobiont Generalist Genes (HGGs) and Specific Plant-Microbiota Regulating Alleles Are Under Natural Selection

A question worth investigating on HGG consists in identifying whether these microbiota-associated loci have been shaped

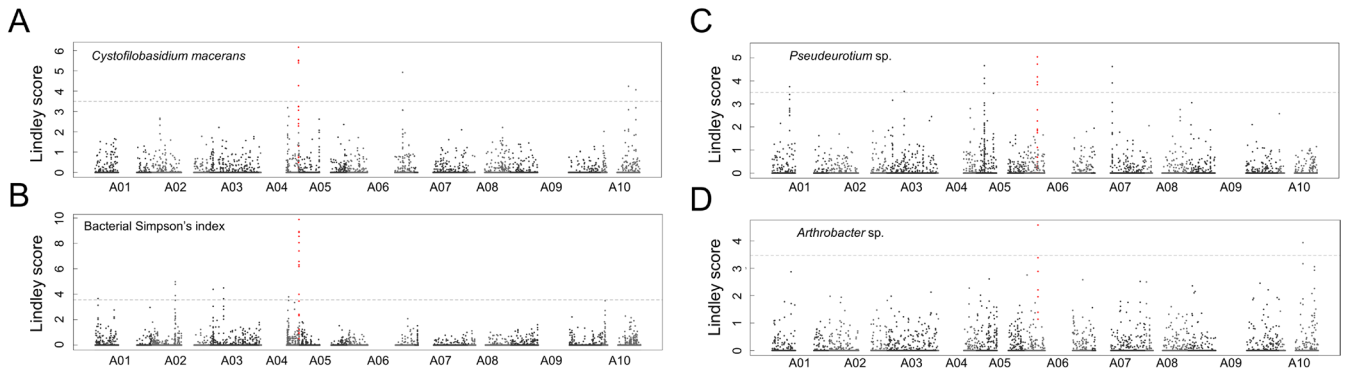


FIGURE 5 | Manhattan plots illustrating genome–environmental analysis (GEA) for holobiont generalist genes (HGGs) found to be associated with both fungal and bacterial descriptors. (A) Manhattan plot for the fungal OTU *Cystofilobasidium macerans* relative abundance estimated in the rhizosphere compartment in spring. The red peak of association on chromosome 4 falls in the A04p30810.1_BraC13 gene coding for a chitinase. (B) Manhattan plot for Simpson's index estimated on the bacterial dataset in fall. The red peak of association in chromosome 4 falls in the A04p30810.1_BraC13 gene. (C) Manhattan plot for the fungal OTU *Pseudeurotium* sp. relative abundance estimated in the root compartment in spring. The red peak of association on chromosome 6 falls in the A06p13350.1_BraC13 gene also called M3KDELTA7. (D) Manhattan plot for the bacterial OTU *Arthrobacter* sp. relative abundance estimated in the rhizosphere compartment in fall. The red peak of association on chromosome 6 falls in the A06p13350.1_BraC13 gene corresponding to M3KDELTA7. For all Manhattan plots, the *y*-axis corresponds to the values of the Lindley process corresponding to the $-\log10\,(p)$ of the tuning parameter $\xi = 3$. The dashed line indicates the significance threshold as described in Bonhomme et al. 2019 (16). Blank spaces indicate centromeric positions that were eliminated from the analysis.

by natural selection. To answer this question, we additionally tested whether the identified SNPs were enriched in a set of SNPs subjected to adaptive spatial differentiation. This was done by estimating a Bayesian measure of spatial genetic differentiation (XtX) (Gautier 2015). We then intersected the top 0.05% SNPs carrying the highest XtX with the top 0.05% SNPs carrying the highest LD values, and we retrieved genes on which these SNPs were located. Among genes shared between the fungal and bacterial descriptors, we found that 6 out of 13 genes shaped signatures of selection; that is, they were associated with high XtX values (Figure 4, Data S13). Within the genes with a strong signature of selection, we found A09p41890.1_BnaRCC (TIP41-like protein *A. thaliana* homologous), A04p30810.1_BnaRCC (chitinase protein *A. thaliana* homologous) and A06p01040.1_BnaRCC (O-fucosyltransferase family protein *A. thaliana* homologous) that are among the alleles shared with the highest number of microbiota descriptors (Figures 4 and 5, Data S13). Funding selection signatures on alleles shared among several microbiome descriptors reinforce the hypothesis that strong selective pressures are acting on plant populations to maintain HGG as they are crucial in modulating the host–microbiome.

Beside HGG, we found strong signatures of selection on alleles that are specific for fungal or bacterial descriptors. Indeed, we identified strong selection signals on A01p08480.1_BnaRCC (Supporting Information Data S13) that are associated with the relative abundance of two fungal species: *Fusicolla* sp. and *Periconia prolifica*, a cobalt-producing fungus with antibacterial activities (Hodhod et al. 2023). The *A. thaliana* ortholog of A01p08480.1_BnaRCC is IBI1 (Impaired in BABA-induced disease Immunity) (Data S13) and acts as a receptor protein activating plant defence through beta-aminobutyric acid (BABA). Interestingly, we found that three *B. rapa* genes (A07p24690.1_BnaRCC, A09p50420.1_BnaRCC and A09p07970.1_BnaRCC) displaying a strong signature of selection were associated with the bacterial biocontrol species *Bacillus simplex* abundance (Data S13). This is coherent with the hypothesis that alleles regulating the presence of beneficial plant–microbes are likely to be positively selected.

3 | Discussion

The holobiont concept is not novel but is recently spreading from the scientific and academic environment to the whole society. On 14th June 2023, 'The Economist' published an article on how holobionts are shifting the biological meaning of an individual https://www.economist.com/science-and-technology/2023/06/14/the-idea-of-holobionts-represents-a-paradigm-shift-in-biology. But how much do we know about the evolution of holobionts? What is obvious is that studying the coevolution between wild plants and the associated microbiomes is an important step in capturing the genetic bases influencing the evolutionary trajectories of holobionts. Here, we take advantage of ecologically and genomically differentiated wild populations of the non-model plant species *B. rapa* to study evolutionary traits of holobionts. Wild *B. rapa* populations (AA, $2n = 2\times = 20$) are closely related to cultivated forms (e.g., turnip, leafy and oilseed crops). They are a unique and understudied natural genetic material, providing invaluable insights into the genetic interactions among holobionts in natural habitats. *B. rapa* wild populations used in our study were identified by flow cytometry, chloroplast genomic regions sequencing and cytogenetic analysis (Falentin et al. 2024) in the context of the H2020 PRIMA project 'BrasExplor'.

Not surprisingly, we found that the *B. rapa* fungal and bacterial microbiome is highly variable among plant compartments (roots and rhizospheres) with the highest diversity in the rhizosphere (Figure 2). This is in accordance with studies conducted on natural *A. thaliana* populations (Bartoli et al. 2018) and several crop species such as maize (Xiong et al. 2012) and other cereals (Michl et al. 2023), or in less-studied plant species like switchgrass (Singer et al. 2018) or *Agave* (Coleman-Derr et al. 2015). As

observed for the bacterial leaf and root microbiome in *A. thaliana* (Bartoli et al. 2018), we found a seasonal effect on the fungal communities that was mostly significant in the root compartment but not in the rhizosphere (Figure 2). On perennial crops, similar results were observed on plant leaves (Howe et al. 2023) with a strong bacterial communities' variation over seasons. It is therefore evident that studying holobionts over several seasons/years and host compartments is crucial to dissect the genomic architecture regulating the host–microbiome interactions. Also, we observed that microbiome variation is not equivalent among fungal and bacterial communities. As shown in (Thiergart et al. 2020) in a reciprocal transplant experiment between Swedish and Italian *A. thaliana* populations, root microbiota variation was primarily influenced by soil origin for bacteria and by location for filamentous eukaryotes, with minimal effects from host genotype. The study also observed strong local adaptation between the two *A. thaliana* populations, suggesting that climate plays a more significant role than soil conditions in plant adaptation and variation in root-associated filamentous eukaryotic communities, while soil properties are primary drivers of bacterial community differentiation in roots. Therefore, different environmental constraints might have a divergent effect on bacterial and/or fungal communities.

In addition, cross-kingdom interactions can influence the evolutionary trajectories of holobionts (Harrison et al. 2021). For example, competition or cooperation among fungal and bacterial communities can define the host–microbiome diversity and composition with direct consequences on the evolutionary interactions within a given holobiont (Bergelson et al. 2019). Therefore, there is an urgent need to take into consideration both prokaryotic and eukaryotic communities when studying holobionts in their native habitats. A genome-wide association study on leaf samples of *A. thaliana* accessions showed a highly differentiated host genetic architecture among fungal and bacterial communities (Horton et al. 2014). Similarly, two studies on *A. thaliana* roots and leaves found almost no overlaps among genes structuring the bacterial and fungal microbiota (Bergelson et al. 2019; Brachi et al. 2022). Nevertheless, the cited studies were conducted in common gardens or experimental fields. The hologenome theory postulates that the evolution of plants and animals is defined by their interactions with the hosted microbiomes in the native ecological context where holobionts coevolve (Moran and Sloan 2015). However, studies of holobionts in their native habitats are still underrepresented. To our knowledge, (Roux et al. 2023) is the only large plant in situ survey describing a highly variable genetic architecture between plant compartments (leaves and roots) and seasons (fall and spring) for the bacterial *A. thaliana* microbiome (Roux et al. 2023). As mentioned by the authors, the next step is to compare fungal and bacterial microbiomes in situ to identify common plant genetic loci structuring inter-kingdom interactions. By controlling for plant population structure (Gautier 2015), our approach on wild *B. rapa* populations allowed the identification of the non-negligent number of 13 HGGs—i.e., genes regulating the holobionts at several layers among prokaryotes and eukaryotes—shared among fungal and bacterial descriptors. As the bacterial communities were not described in the root compartment, it might be possible that this number is underestimated. Also, underestimation of HGG can be related to important population plant structure within the French and Italian populations. As the Bayesian model used in our study corrects for population structure, true positive associations might be removed by decreasing the number of HGG on the overall analysis.

Similarly, our study focuses on 26 wild populations, and including more plant genetic diversity could increase the chance to individuate a larger number of HGG. However, 'actual' *B. rapa* wild populations are scarcely distributed and hard to detect and taxonomically identify. Therefore, increasing the number of plant populations is not as easy as for the worldwide distributed and amply studied *A. thaliana*.

Among the HGG, a large part was associated with fungal or bacterial OTUs reported to be biocontrol agents or plant-growth-promoting (PGP) microbes. For instance, A06p13350.1_BnaRCC was associated with: (i) the fungal species *Pseudeurotium* sp. known to be a tomato endophyte with both PGP and biocontrol activity (Manzotti et al. 2020) and (ii) with the promising biocontrol *Arthrobacter* bacterial species (Figure 5) enhancing plant growth and known to suppress mould disease in tomato (Roy and Kumar 2020; Ramlawi et al. 2021). The A06p13350.1_BnaRCC *A. thaliana* homologous (AT1G18160) belonging to the M3KDELTA7 family was described to be required for the ABA signal transduction via SnRK2 (snf1-related protein kinase2s) activation. ABA is known to play a crucial role in plant development as well as in stress tolerance (Kavi Kishor et al. 2022). Therefore, in natural plant populations, beneficial fungal and bacterial communities are expected to trigger ABA host responses to confer resistance to both biotic (as pathogens' attacks) and abiotic stresses. Alleles recognising beneficial microbes are then likely to be positively selected in natural plant populations. Nevertheless, the A06p13350.1_BnaRCC was not found in our study to be under selection (Figure 4). On the other hand, the A09p41890.1_BnaRCC gene associated with both *Preussia* fungus and *Bacillus simplex* biocontrol bacterium (Shafi et al. 2017) was enriched for XtX suggesting that this gene is under natural selection in the *B. rapa* populations described here. *Preussia* sp. is producing nitric oxide (NO) and phytohormones like indole-3-acetic acid (IAA) and several gibberellins, and was shown to improve rice growth under greenhouse conditions (Al-Hosni et al. 2018). As for the M3KDELTA7 family, the A06p13350.1_BnaRCC *A. thaliana* ortholog is coding a TIP41 like-protein inducing ABA responses (Punzo et al. 2018). This suggests that HGG not only influence several plant beneficial microbes, but they also trigger generalist plant stress defence pathways. Holobionts are therefore likely to share common biological mechanisms that concretely act to protect the host by positively selecting for microbes that can enhance immune defences or stimulate host growth.

However, we need to be cautious in interpreting the conclusions of our study, as GEA allows us to identify correlations between host genetic variability and environmental variables, such as microbiomes, but does not determine whether these correlations reflect true biological interactions or mere associations. Therefore, the next step is to validate these HGG, as has been done for other plant genes correlated with the abundance of beneficial microbes. To achieve this validation, gene knockouts, transcriptomic analyses, or experimental microbiome manipulations (such as SynComs reconstruction) are necessary to confirm the biological roles of these candidate genes. For instance,

by establishing fungal and bacterial collections from natural plant populations, the next step is to validate the role of HGG in interactions with native microbes (isolated from the original plant populations) to explore their function in modulating host defence responses. It is worth underlining that validating the role of plant genes in modulating microbial-associated communities should integrate several approaches, such as (i) longitudinal and multi-generational studies to track microbiota inheritance and host evolution, (ii) experimental evolution with gnotobiotic models (germ-free plants colonised with SynComs) to determine whether microbiome changes influence genetic evolution, (iii) host–microbiome coevolution in wider natural populations, examining whether microbiota influences adaptation to environmental pressures.

4 | Material and Methods

4.1 | Plant Sampling and Genome Sequencing and Analysis

To perform plant genomic characterisation, 26 populations were collected during February 2020 in the south of Italy (Sicily) and during May 2020 in the north of France (Brittany and Pays de Loire, DataSet S1). Plants were collected at different times in northern France and Italy but at the same vegetative stage. Indeed, all Italian and French populations were collected at the bolting-time stage. We therefore aimed to collect plants at an equal vegetative and developmental stage rather than in the same month. As spring arrives earlier in southern Italy compared to northern France, *B. rapa* populations also germinate earlier. For plant PoolSeq sequencing, leaves from 30 individuals within each plant population were collected directly in situ, and an equal leaf stab was pooled into a sterilised 50 mL Falcon tube that was stored at −80°C for 24 h prior to 2 days of lyophilisation. Twenty-five milligrams of lyophilised leaves were then placed into a 2-mL Eppendorf tube and homogenised for 1 min at 35 Hz with the mixer mill RETSCH MM 400. DNA of the lyophilised leaves was extracted with the Qiagen DNeasy Plant Pro Kit by following the manufacturer's instructions. Integrity, purity and quantity of the DNA were assessed by running 5 μL of the extracted DNA on a 1% agarose gel and by Thermo Scientific NanoDrop spectrophotometer. Plant DNA was sequenced at the BGI platform (Hong-Kong, China). For this, 1 μg of genomic DNA was randomly fragmented with Covaris. DNA fragments were selected by Agencourt AMPure XP-Medium kit to an average size of 200–400 bp. Fragments were end repaired and then 3′ adenylated. Adaptors were ligated to the ends of these 3′ adenylated fragments. Fragments were then amplified by PCR followed by a purification with Agencourt AMPure XP-Medium kit. The double stranded PCR products were heat denatured and circularised by the splint oligosequence. The single strand circle DNA (ssCir DNA) was formatted as the final library. Libraries were qualified by QC. The qualified libraries were sequenced by BGISEQ-500: ssCir DNA molecules formed a DNA nanoball (DNB) containing more than 300 copies through rolling-circle replication. The DNBs were loaded into the patterned nanoarray by using high density DNA nanochip technology. Finally, 100 bp paired end reads were obtained by combinatorial probe-anchor synthesis (cPAS). Raw plant reads were trimmed using Fastp v0.20.1 (Chen et al. 2018; Li 2013). Clean reads were then mapped to *B. rapa* C1.3 genome assembly (Supporting Information Text) using bwa mem v0.7.17-r1188 and SNP calling was conducted with varscan mpileup2cns v2.4.4 https://dkobo ldt.github.io/varscan/. The plant allele frequencies matrix was trimmed according to four successive criteria: (i) removing SNPs with missing values in more than two populations, (ii) only sites with mean depth values (over all populations) greater than or equal to the mean depth minus the standard deviation for depth and less than the mean depth plus the standard deviation for depth were kept, (iii) sites with a minor allele frequency (MAF) under 0.05 were removed and (iv) SNPs falling into centromeric regions were deleted.

4.2 | In Situ Microbiota Characterisation

For *B. rapa* microbiota characterisation, roots and rhizosphere samples were collected and separated in situ and stored in a cold box at 4°C during travelling. In total, we collected six plants for each population during two seasons: (i) spring, corresponding to February 2020 in Italy and May 2020 in France, and (ii) fall, corresponding to December 2020 in Italy and January 2021 in France. The six plants were collected at different spatial distributions to cover the entire plant population. This was done to represent the maximum of the diversity present in the population. As we explained above, plants were collected at the same vegetative stage instead of at the same month because climatic patterns between the two countries affect plant germination and development. In spring, both Italian and French populations were at the bolting-time stage and in fall at the five-leave stage. Each root rhizosphere sample was placed in a 2-mL Eppendorf tube containing 3-mm metallic beads and it was lyophilised for 2 days. After lyophilisation, all samples were homogenised into individual tubes for 1 min at 35 Hz with the mixer mill RETSCH MM 400. The obtained powders were placed in 1.2-mLS-block 96-well plates by using a metallic spoon. At each sample, the spoon was cleaned with pure alcohol. DNA was extracted by using the Qiagen DNeasy 96 PowerSoil Pro Kit (reference: 47017) by following the kit instructions. The extracted DNA was then quantified with the NanoDrop spectrophotometer. All DNAs were adjusted to 20 ng/μl prior to amplicon generation. Specifically, bacterial communities were characterised through amplification of a fraction of the *rpoB* gene with primers and conditions described before (Ogier et al. 2019) by using internal TAGS for multiplexing as described in Bartoli et al. (2018). Fungal communities were characterised by amplifying a portion of the ITS1 with a protocol developed in this study and adapted to *B. rapa*. For this, the following primers have been used: ITS1Fngs 5′—GGTCATTTAGAGGAAGTAA—3′ and ITS2ngs 5′—TTYRCKRCGTTCTTCATCG—3′. The′. PCR program consisted of: initial denaturation at 95°C for 5 min, 35 cycles at 95°C for 5 s, 58°C for 30 s, 72°C for 30 s and a final elongation at 72°C for 10 min. All PCRs for both markers were performed in a final volume of 50 μL with the MTP Taq DNA Polymerase (Sigma-Aldrich, reference: D7442). PCR amplicons were then purified by using AgencourtAMPure magnetic beads following the manufacturer's instructions. Purified amplicons were quantified by spectrometry and appropriately diluted to obtain an equimolar concentration. Two microliters of equimolar PCR-purified products were used for a second PCR containing the 288 Illumina indexes. The second PCR amplicons were then

purified and quantified as described above to obtain a unique equimolar pool that was quantified by real-time quantitative reverse transcriptase-polymerase chain reaction and sequenced with Illumina MiSeq 2×250 v3 (Illumina Inc. San Diego, CA, USA) in the GeT-PlaGe Platform (Toulouse, France).

4.3 | Microbiota Analysis

4.3.1 | Bioinformatics Analysis and Data Filtering

Raw reads were demultiplexed by considering the Illumina indexes and the three internal TAGS with the Flexbar software available at https://github.com/seqan/flexbar. For the fungal communities, the total number of reads and OTUs before data trimming was 11 345 800 and 14 142 respectively. The average number of complete pairs sequences per sample was 18 182 (SD = 5, 022). For the bacterial data set, the total number of reads and OTUs before data trimming was 3 631 685 and 6 838 respectively. The average number of complete pairs per sample was 1 125 (SD = 963). For both *rpoB* and ITS1 datasets, we removed samples with less than 1000 reads to keep only samples represented by a correct number of reads. This led to the elimination of the entire *rpoB* roots dataset. Taxonomic affiliation was performed by using a Bayesian classifier implemented in the classify.seqs command of Mothur (Schloss et al. 2009) against the UNITE ITS database v 8.2 (fungi-only) and the *rpoB* database available at FROGS https://frogs.toulouse.inra.fr/. Clustering of sequences into OTUs was performed with Swarm (Mahé et al. 2015) https://github.com/torognes/swarm by using a clustering threshold (d) = 1. Only OTUs with a minimum of five sequences across all samples were kept. After data filtering, for the ITS we obtained 602 samples and 1227 OTUs, and for *rpoB* 465 samples and 1237 OTUs. Scripts for bioinformatic analysis are provided in the Supporting Information under the script section.

4.3.2 | Analysis of Microbiota Descriptors

The final OTU matrices obtained after data filtering were used to estimate microbiota α-diversity descriptors with summary. single function in Mothur (Schloss et al. 2009). Microbiota composition (β-diversity) was inferred and analysed with two complementary approaches. Firstly, a Hellinger transformation was performed by using the vegan R package and the relative Hellinger distance was inferred with the decostand command in the vegan R package prior to β-diversity analyses. For both fungal and bacterial datasets, the Hellinger distance matrices were reduced by running a PCoA with the ape R package. Secondly, we applied constrained ordination (tb-RDA) on the OTU matrices. For this, we first estimated the relative abundance of each bacterial and fungal OTU, and selected OTUs with a frequency ≥1. The trimmed OTU count table was then normalised and tb-RDA inferred with the rda function in the vegan R package. To control for plant compartment, tb-RDA was independently run on rhizosphere and root samples. Natural variation for microbiota descriptors for both fungal and bacterial communities was used to run linear-mixed models with the lmer function in the lsmeans R package and lm4 by considering the plant compartment and genotype as fixed factors in the model. p-values obtained by linear-mixed analysis were corrected for the FDR. As both fungal and bacterial communities were dependent on the plant compartment, linear-mixed models were inferred separately on rhizosphere and root samples.

Box plots on microbiota diversity descriptors were performed in the R environment by using general coding (scripts available in Supporting Information). To visualise the seasonal effect on α-diversity descriptors, we plotted Shannon and richness data for both fungal and bacterial communities in a Finlay Wilkinson by using ggplot2. To test the impact of plant population on the variation of fungal and bacterial microbiota descriptors, we performed broad-sense heritability (H^2) by lmer model from the lme4 package, and by considering the plant population as a random effect. H^2 was then estimated by dividing the variance obtained for the plant genotype effect by the sum of the genotype variance and the residual variance that was divided by the number of replicates in the dataset. As H^2 showed a high effect of the plant population on microbiota descriptors, we estimated BLUPs by running a linear model with lmer and by considering the plant population as a fixed effect. BLUPs were estimated for species richness, Shannon's index, the first four PCoA axis, and the most relevant, but variable among the plant populations, fungal and bacterial OTUs, which are present in at least 10 samples. As we found a seasonal and plant compartment effect, BLUPs were individually estimated for each combination within the fungal and bacterial descriptors: R-FA (roots in fall), R-SP (roots in spring), RH-FA (rhizosphere in fall) and RH-SP (rhizosphere in spring). Script for statistical analysis and figure's construction are provided below in the script section.

The list of fungal and bacterial OTUs obtained after data trimming and the corresponding taxonomy and sequences are available in Dataset S14 and S15 respectively.

4.4 | Genome–Environmental Analysis (GEA) to Finely Map Plant Loci to Microbiota Descriptors

GEA analysis was performed on microbiota descriptors (species richness, Shannon's diversity, PCoA first four axes and abundance of the most prevalent fungal and bacterial OTUs) as described in Roux et al. (2023) and Frachon et al. (2023). Detailed information about GEA analysis and related scripts is provided in Supporting Information Text. Microbiota descriptors used for GEA analysis are available in Dataset S4–S10.

Author Contributions

Loeiz Maillet: writing – original draft, formal analysis, data curation, conceptualization, visualization, methodology. **Manon Norest:** methodology, data curation. **Adam Kautsky:** methodology, data curation. **Anna Geraci:** methodology. **Elisabetta Oddo:** methodology. **Angelo Troia:** methodology. **Anne-Yvonne Guillerm-Erckelboudt:** methodology. **Cyril Falentin:** methodology. **Mathieu Rousseau-Gueutin:** methodology. **Anne-Marie Chèvre:** methodology. **Benjamin Istace:** methodology. **Corinne Cruaud:** methodology. **Caroline Belser:** methodology. **Jean-Marc Aury:** methodology. **Rosario Schicchi:** investigation. **Léa Frachon:** methodology, investigation. **Claudia Bartoli:** conceptualization, methodology, investigation, funding acquisition, writing – original draft, writing – review and editing, validation, supervision, resources, project administration, visualization.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

Raw FastQ reads corresponding to the microbiota ITS and *rpoB* characterisation were deposited in the sequence read archive (SRA) of NCBI and are available at https://www.ncbi.nlm.nih.gov/sra/PRJNA1049637. Plant raw reads were deposited on NCBI and are available under the BioProject number PRJNA1049637 and the BioSample accession listed in Dataset S16. Reads, genome assembly and gene prediction of *Brassica napus* RCC-S0 are available in the European Nucleotide Archive under the following project PRJEB71115. OTU taxonomic affiliations are reported in Dataset S14 for fungal communities and Dataset S15 for bacterial communities. Scripts used for bioinformatic and statistical analysis are reported in the Supporting Information Text. Genetic markers of *Brassica napus* RCCS0 genome are reported in Dataset S17.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.