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Review paper

Can functional hologenomics aid tackling current challenges in plant breeding?

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Abstract

Molecular plant breeding usually overlooks the genetic variability that arises from the association of plants with endophytic microorganisms, when looking at agronomic interesting target traits. This source of variability can have crucial effects on the functionality of the organism considered as a whole (the holobiont), and therefore can be selectable in breeding programs. However, seeing the holobiont as a unit for selection and improvement in breeding programs requires novel approaches for genotyping and phenotyping. These should not focus just at the plant level, but also include the associated endophytes and their functional effects on the plant, to make effective desirable trait screenings. The present review intends to draw attention to a new research field on functional hologenomics that if associated with adequate phenotyping tools could greatly increase the efficiency of breeding programs.

Key words: endophytes; holobiont; molecular markers; genetic variability; plant functionality; phenotype

Introduction

The most basic assumption, on which plant breeding relies, is the relationship between phenotype and genotype. In conventional breeding, high correspondence of genotype and phenotype implies negligible effects of the environment to heritability of the trait. That is often not the case, and it is hoped that the emergence of phenomics [1] will enable a fine resolution of genetic and environmental parts of inheritance. This is

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one of the primary aims of functional genomics in agricultural species—to connect genotype to phenotype and use this knowledge to make phenotypic predictions and select improved plant types with specific desired traits.

To understand the genotype–phenotype relation, we do need to realize that life is organized in a hierarchical fashion. Genes are organized on chromosomes, chromosomes in nuclei, organelles in cells, cells in individuals and individuals of different species in symbioses. Entities thus form groups that can become a new unit of selection. It is estimated that >20 000 species of plants are obligatorily dependent on microbial cooperation for development, growth and survival [2], and crop plants are no exception. How does considering plant–symbiotic microorganisms as an entity challenge the current views in plant breeding and, in particular, the use of recent and emerging technologies for genotype screening and phenotype prediction (such as whole genome and/or transcriptome sequencing, genotyping by sequencing, molecular marker discovery, association mapping, gene mining)? Recently, Arnholdt-Schmitt and co-workers [3] call attention to the fact that most organisms exist as 'superorganisms' or 'holobionts' [4–7], which challenges functional marker development in plant breeding, and that the complexity can potentially be handled through the correct use of efficient tools for measuring 'effects' in the target tissues for final traits. The present manuscript starts from that perspective and goes further, bringing forward a functional hologenomics view in plant breeding. It intends to stimulate debate and to encourage reconsideration of some of the rationalities traditionally used in plant breeding, particularly when selecting for agronomic target traits.

Endophytes and the hologenome concept in plant breeding

All plants known to date, regardless of their natural ecosystems, live in association with microorganisms [8–10]. Studies on the microbiology of plants have shown that endophytes usually colonize different compartments of the plant apoplast, including the intercellular spaces of the cell walls, xylem vessels and intracellular plant tissues [11–15]. These microorganisms can be also found in three locations: around the roots (rhizosphere), on the leaves, stems, flowers, fruits (phyllosphere) and seeds (for an extensive review see [7]). Endophytes have been traditionally defined as: 'fungi or bacteria which, for all or part of their life cycle, invade the tissues of living plants and cause unapparent and asymptomatic infections entirely within plant tissues but cause no symptoms of disease' [16, 17], or, for a broader definition, we can consider endophytes as microorganisms, often fungi or bacteria, that live in association with plants [17, 18]. This last definition acknowledges the fact that the net effects of such microorganisms on the host are highly conditional and range from mutualism to antagonism. Throughout the present article, we will be considering endophytes as symbiotic microorganisms, including fungi, bacteria, virus and algae, that develop within the plant tissues, and holobiont (following [7, 19]) as the plant host and its entire symbiont population.

Endophytes can be transmitted from the parents to the offspring—vertical transmission—or can be acquired from the environment—horizontal transmission. Whereas vertical transmission entails a stronger co-evolution, with host–symbiont association being kept throughout generations [7, 20, 21], horizontal transmission implies—by definition—that new host–symbiont combinations can be formed unless other mechanisms are in force to ensure host–symbiont specificity (e.g. synchronization to lifecycles, host–symbiont recognition, partner fidelity feedback). Douglas [22] claimed that in symbioses with horizontal transmission hosts can generally form associations with a broad range of symbionts, including taxa from which they derive little or no benefit. Dominant endophytic taxa, such as *Alternaria*, *Cladosporium* and *Epicoccum*, seem not to be host specific and are opportunistic colonizers of many plants [23], albeit the evidence of conferring host resistance to insect herbivores and pathogens [24–26]. Endophytic symbionts penetrate the plant host through stomata, nectarthodes, lenticels, germinating radicles, tissue wounds associated with the emergence of secondary roots, broken trichomes, foliar damages caused by soil particles, rain or hail or through undifferentiated meristematic root tissue [27]. Once in the plant, they can affect the functionality of the host through their influence on its phenotype and epigenome [28, 29].

Plant microbiome can be thus one of the key determinants of plant health and productivity [10] because of its essential role in plant phenotypic and epigenetic plasticity [28–34]. Many studies have shown the potential of the use of endophytes in agriculture and how they change the functionality of different host plant genotypes to better respond to particular agronomic requirements [30]. Endophytes can help plants: to suppress diseases by competing for space with pathogens or by inducing plant stress resistance response [35–37], to stimulate growth through production of phytohormones [37, 38] as well as withstanding abiotic stresses such as heat [39], drought [40] and salt [41, 42]. Endophytes can influence crop yield and quality by nutrient mobilization and transport, especially minerals present in the soil that might be otherwise inaccessible to plants [43, 44]. However, to our knowledge, no studies have systematically attempted to explore the potential of endophytes as standing variability that directly affect important and vital traits of the plant. In this way, they can constitute a major new source of selectable variability with expected impacts in breeding strategies. Indeed, all functional effects of endophytes on the plant host are mediated by changes in its gene expression, representing thus an 'extended phenotype' of the microorganisms to which it is associated. The perceivable 'plant phenotype' is thus the product of concerted and co-regulated expression of both plant and microbial genes, together with environmental influences. Rosenberg *et al*. [19] stresses that the holobiont—in this case plant and associated microorganisms—with its hologenome, is the unit of natural selection in evolution.

The holobiont is defined as the host organism and all its associated symbiotic microbes, including parasites, mutualists, synergists and amensalists [4, 7, 20, 45], being the hologenome the summation of the genetic information of the host and its microbiota [46, 47]. If nature is selecting at the level of holobiont and hologenome rather than individuals or genomes, variation—the raw material for evolution—can arise from changes in either the host or the symbiont microbiome—endophytic microbiome—or both, and be transmitted from one generation to the next with fidelity [23]. In such a frame, the symbiotic microbial community, which can change more rapidly, would help the holobiont in surviving and thus gathering the necessary time for the host genome to evolve (which has typically slower evolution rates)—and the holobiont to adapt and evolve [47]. Recently, Soen [48] argued that microbial changes provide a potential infrastructure for causal links between immediate responses to new environments and longer-term establishment of evolutionary adaptations.

Until recently, this hidden microbial world was so little recognized in the context of plant breeding (with some specific

exceptions that profoundly affect plant productivity and feeding value such as fungal toxicities—the classical example is grasses for cattle [49–51]—plant diseases, legume–rhizobia symbiosis or plant–mycorrhizal fungus interactions). The reason seems obvious: perception of the microbial world usually requires observation of the microorganisms (often not cultivable in laboratory conditions) and/or consequences of their activities, which can be subtle and difficult to investigate experimentally. However, there is increasing appreciation that microbes are an essential part of the host's phenotype and that they have a high influence on fitness and other ecologically important traits [7, 35, 36, 41, 44]. The fast development of molecular techniques (specially the ultrahigh-throughput sequencing methods) during the past years has made now feasible the *in situ* detection of microorganisms (reviewed in [52]), as well as the extensive genomic and transcriptomic analyses of entire microbial communities (metagenomics and metatranscriptomics) [53]. Nevertheless, in this new 'omics era', where a new set of tools and techniques allowing the study of the holobiont are available, the focus remains mainly on the plant side, overestimating by this way the contribution of the plant's genotype to the overall phenotype in breeding populations. The fact that the observed phenotype is the phenotype of the holobiont (as opposing to the plant alone) is still generally not considered.

Genomics-assisted breeding profits from considering the hologenome

Genomics-assisted breeding refers to the integration and use of genomic tools—such as genomics, transcriptomics and proteomics—in breeding programs for developing lines with enhanced biotic or abiotic stress tolerance and improved yield. Also through the identification of molecular markers that associate with traits of interest, genomics-assisted breeding helps breeders to predict the phenotype from the genotype. By considering the hologenome, we are likely to get a more consistent coupling between genotype and phenotype.

Advances in genetics and genomics have greatly enhanced our understanding of structural and functional aspects of plant genomes, increasing the basic knowledge and its integration towards tackling one of the biggest challenges in this area: identification of the genes underlying a trait of interest (gene mining), so they can be exploited in crop improvement [54]. New genomic techniques allow studying the whole genome and transcriptome in a cost-efficient way. In particular, the development of high-throughput DNA sequencing technologies has become one of the main pillars of genomic breeding. These techniques have enabled to create genome-wide molecular tools for breeders (large collections of markers, high-throughput genotyping strategies, high-density genetic maps, new experimental populations, etc.) that have been incorporated into already existing breeding methods [55–58] to improve and accelerate the breeding process in many ways (amongst others association mapping, marker-assisted selection (MAS), 'breeding by design', gene pyramiding, genomic selection, etc. [58–62]). However, failure to apply those techniques and approaches in the most comprehensive way might compromise an identified association between genotype and phenotype. The genetic marker coupling to a given phenotype likely does not stand in all environmental situations and in the presence of different endophytes; therefore the utility of a given tool for plant breeding ends up to be limited.

Also, when considering genome engineering programs, the presence of endophytes can impact the outcome. These new tools of genetic/genomic engineering have made possible the transfer of genes among diverse species, and plant transformation has become an important mean by which crops are improved. It has been shown that genome editing can accelerate plant breeding by allowing the introduction of precise gene modifications or insertions directly in an elite background [63]. They can be used to eliminate genes that negatively affect food quality, or that confer susceptibility to pathogens [63, 64], and also to generate disease resistance [65], for example. However, phenotypic outcomes of targeted modifications are seldom predictable and depend on the environmental conditions and on the endophytic community. Some plants have been transformed with genetic material containing genes coding for compounds, such as antimicrobial agents, that could affect not only the desired targets such as plant pathogens, insects or herbicide resistance, but also nitrogen-fixing bacteria, mycorrhizal fungi and other beneficial soil microorganisms and alter their interaction with host plants [66–68], which can have unpredicted effects on the plants' fitness or could affect the functionality of the plant under different environmental situations.

It is unarguable by now that endophytes affect plants' functionality, and that the association with different endophytes confer different characteristics to their host. It is thus expected that one particular plant genotype (engineered or not) may give rise not only to one predictable phenotype, but to a range of unpredicted phenotypes depending on the associated microbiome [30, 69–71]. Furthermore, as plant-associated endophytic microorganisms affect important and vital traits of the plant, they can provide a new source of selectable variability. Thus, understanding interactions between plants and endophytes, identifying the plant alleles controlling them as well as the molecular mechanisms underlying phenotypic traits at plant level [72] can have large repercussions in plant breeding.

Higher genetic variability in crops than previously believed

Plant breeding requires genetic variability as the raw material for selection to increase the frequencies of favorable alleles and genetic combinations. Sources of genetic variability can be found within the crop, mostly in the form of landraces and also within crop wild relatives [73]. However, as a consequence of the selection processes during historical domestication and adaptation of crop plants, a considerable loss of diversity has occurred, and the variability that breeders have to work with in modern breeding populations is limited. Detecting genetic variability within natural and breeding populations is crucial for effective utilization of the genetic resources available, and there is a need to elucidate the causative genetic differences that give rise to observed phenotypic variation. Breeders are constantly in quest of new sources of genetic variation and have been successful in identifying them whenever advances in scientific knowledge and novel technologies permitted, such as epigenetic variation as a source of selectable epialleles in breeding [74]. There is yet another source of variability that has been traditionally neglected in plant breeding and that advent technologies allow to explore and exploit the direct variability resulting from crop-associated endophytes.

The hologenome can change either at the host or at the endophyte component. Allelic variation can thus arise from recombination and mutation (as is commonly considered for the plant host only). Whereas for the plants, recombination implies sexual reproduction and/or chromosome rearrangements, for

the microorganisms, it can occur in different ways (e.g. in haploid bacteria, within-species recombination occurs by conjugation, transduction and DNA transformation, and between-species by horizontal gene transfer), and mutation rates are estimated much higher in microorganisms [75]. Two other sources of variation are relevant and potentially causing changes to the hologenome: symbiont population changes in numbers (but not in type, being equivalent to gene amplification [76]) and acquisition of novel endophytic symbionts (and thus new genes) from the environment. Whereas the last source of variation is too much dependent on the environment, and a higher challenge to be taken into consideration, the former can—and to our understanding should—be considered when identifying useful variation for breeding programs. With today's tools, hapmaps—haplotype maps of entire collections useful to identify rare, potentially valuable, alleles—could be extended to include endophyte data, at least as a variable. The genetic variation conferred by some endophytes can be transmitted to offspring and is thus potentially selectable. Agricultural practices and breeding programs have the potential to select for higher or lower symbiotic effectiveness [77, 78], and the outcome depends on the awareness of the microbiome and its influence on traits of interest and on an evolutionarily informed approach.

Studying genetic variation of crops, landraces and crop wild relatives on a hologenome-wide scale seems to be a rational step forward for assessing existing diversity, characterizing populations and providing a deeper insight into the mechanisms of regulatory evolution that act on the holobiont. In sum, there is more variability to select from than one can think if considering the plant host alone.

Challenges in marker assisted selection

Weak associations between genotype and phenotype

Once the reference genome of a crop is sequenced and assembled, multiple individuals within species can be sequenced and genetic variants can be detected in a more cost-efficient way. Available already nowadays are large marker collections and high-resolution maps, which have greatly increased the accuracy and resolution of quantitative trace loci (QTL) and association mapping studies [73]. This knowledge has had a large impact into crop improvement. Marker assisted selection (MAS) is a process commonly used in plant breeding, whereby selection is carried out on the basis of a marker (or a set of markers) instead of the trait itself. The successful application of MAS relies thus on the tight association between the phenotype and the marker, and therefore, identification of marker–trait associations is the first critical step for it. So far, traits that display simple inheritance or QTLs that explain a substantial portion of the phenotypic variation have been used in MAS [79]. However, in some complex agronomic traits such as drought tolerance, disease resistance and yield stability, breeders find that markers or QTLs identified in a particular mapping population are not effective in all different backgrounds [60, 80] and may vary considerably in magnitude across environments [60 and references therein]. These weak associations between plant genotype and phenotype are—rightfully—attributed to the complexity of quantitative traits: those traits are controlled by many genes with small effects, show large epistatic effects, or are strongly influenced by the environment, and can have low to moderate heritability [81–83]. In such cases, DNA-based markers do not properly predict phenotypes, as these enable to assess the potential of a particular genotype to develop a particular phenotype, but provide no information on the actual metabolic processes occurring in plants on a particular environment [84].

Crop yield stability, for example, is one of the most complex traits in agriculture. The terms 'stability' or 'adaptability' refer to consistent high performance of genotypes across diverse types of environments [85]. It is a measure of how reliable a genotype performs across different growing seasons and locations. A stable genotype is less affected by genotype × environment (GxE) interactions. However, only a minor part of the GxE interaction can be attributed to known environmental determinants, while the major part is a quantity derived from statistical analysis of yield trials that cannot be assigned to known constituents. These unexplained variations in yield refer to fluctuations in the phenotypic expression of yield, while the genotypic composition of the varieties or populations remains stable [86]. It is likely that endophyte community might account for a high degree of this unexplained variability, and therefore, higher stability of performance is to be achieved when selection is performed under the holobiont concept, using appropriately designed molecular tools. Endophytes can indeed be responsible for quantitative variation of host's yield: e.g. in *Taxus* species, taxol levels (an anticancer drug) vary considerably from tree to tree, and this is because of a correlation between plant taxol content and the quantity of its taxol-producing fungal endophyte; the fungal endophyte was found to affect plant taxol yield by eliciting transcription of rate-limiting genes in the plant taxol biosynthetic pathway [87]. How applicable is this model in other plant–endophyte interactions remains to be investigated. However, it is thus likely that endophyte community accounts for a high degree of the unexplained variability, and therefore, higher stability of performance may be achieved when selection methods take into consideration the holobiont concept, using appropriately designed molecular tools.

The manifestation of quantitative traits and the markers developed to predict important agronomic traits can be affected by the endophyte community because the plant-associated microbiome has the potential to change the genetic background, thus compromising the DNA marker. Besides, it is also known that the magnitude and direction of the effect of a given endophyte community can vary between environmental conditions and host genotype, further challenging the prediction/measurement of the effects. For example, in traits such as drought tolerance, association analysis between genotyping data and phenotypes showed that majority of the QTLs and markers encountered by genome-wide association studies (GWAS) contributed relatively little to phenotypic variation in drought tolerance of legume species [88–91]. In this specific case, we are aware that those legume species live in symbiosis with rhizobia that confer different levels of tolerance against abiotic stresses to the plant [92, 93]. If the putative presence of endophytes with known effects on improving drought tolerance is not considered when phenotyping, even though their potential to change gene expression profiles and the physiology of their hosts—and thus the observed trait—is well known, the identified QTLs and markers might reflect a non-stable relation between the gene(s) and the trait. Knowledge on the holobiont could allow studying the trait under different symbiotic states, and understanding its plasticity. Genotype by endophyte interaction (and its stability across environments) could be accounted for and assessed in plant-breeding programs to identify the most efficient communities.

We suggest that some of the weakness in the plant genotype to phenotype association could be improved by (i) considering

endophytes as part of the phenotype, and (ii) considering the hologenome for marker discovery. Endophytes affect the functionality of the host plant by changing its phenotype and epigenome; GWAS and QTL mapping, neglecting this component, are overlooking trait complexity and often result in shallow associations of reduced applicability for breeding. Traits where endophytes are already known to have large influence—e.g. drought tolerance [94–98], nitrogen use efficiency-related traits [99, 100], pathogen resistance [36, 101, 102]—and important traits such as yield stability where they are expected to have also an enormous influence, are the best candidates for a hologenomic and holobiont approach.

Arnholdt-Schmitt *et al.* [3] proposed an experimental step-by-step approach for considering the existence of holobionts in functional marker development, which includes criteria-complex selection of upstream-candidate gene(s), and consecutively, tool development parameters for deep phenotyping. Current phenotyping procedures for evaluating one or more traits involve visual assessment of agronomic traits or resistance to biotic or abiotic stresses in field or greenhouse conditions, as well as laboratory tests [60]. As discussed before, the observed phenotype is the holobiont phenotype [7], and thus the endophytic community needs to be characterized to some extent. Here, a core-microbiome should be considered (long-lasting interactions excluding thus transitory associations), and the challenge is this in defining it to a significant dimension (recently discussed in [7]). The characterization of this microbiome need not to be extensive (i.e. can vary depending on the specific aim), but the effect measuring tool should consider its existence, so that the trait can be more accurately predicted [3]. Considering endophytic community has the potential to ameliorate the predictive capability of DNA-based markers, likely increasing its robustness to different genetic backgrounds or environmental conditions.

Molecular markers based on advantageous alleles or traits on endophytic microorganisms and other ones based on optimal plant responsiveness to beneficial endophytic associations could be developed to assist screenings on desired agronomic performance of the holobiont. This knowledge could potentially allow manipulating the microbiome toward commercially profitable phenotypes.

Bringing functional metagenomics into plant breeding

The nowadays available genomic tools make possible the characterization of the endophytes associated with each particular plant genotype under different environmental conditions. To date, most studies have focused on characterizing plant-associated microbiomes on the rhizosphere [103–105]. For example, by pyrosequencing neutral marker 16S rRNA gene amplicons, Peiffer *et al.* [72] characterized the rhizosphere microbial community composition across a genetically diverse collection of modern maize inbreds in five agricultural field environments. They clearly showed evidence of heritable variation in rhizosphere microbial community composition and considerable field-specific heritable variation. Besides the rhizosphere, only few other compartments have been studied in this respect [106]. High-throughput sequencing is extending our knowledge of plant microbiome diversity, but sequencing, assembling and analyzing a holobiont remains a challenging task. Metagenomics can generate an enormous volume of data sets, demanding highly efficient algorithms (within current computational power); the query sequences that originate from endophytic organisms in a sample often lack taxonomically related sequences in existing reference databases. A first step would be identifying a host plant core-microbiome alone—the stable, consistent components across complex assemblages. Attempts to get to core microbiomes were made in different organisms [107–109].

Metagenomics can, nonetheless, be implemented for assessing genetic variability of plant endophytes and, when focusing on functionality, can select for desired beneficial traits among the endophyte populations that inhabit the host [27]. Indeed, with the development of metagenomics, novel genes, gene products and biological motifs have been discovered, and its functional analysis has been made possible [110]. Currently, genomic resources including genomic and cDNA libraries, microarrays, web-based bioinformatic portals and annotation and gene expression databases are available and becoming more and more comprehensive. Well-resolved phylogenetic frameworks for plant endophytic organisms are starting to appear. Examples are for the *Azoarcus* sp strain BH72 [111], *Piriformospora indica* [112], *Enterobacter radicincitans* [113], *Burkholderia* sp. strain KJ006 [114] and *Variovorax paradoxus* S110 [115]; these frameworks can contribute greatly to the study of endophyte traits involved in plant growth promotion and plant protection (e.g. increased ecological fitness and competitiveness in the rhizosphere, better root and soil colonization ability and enhanced capabilities for suppressing plant diseases). Let us consider the example of iron uptake: the production of siderophores by endophytic *Pseudomonas* spp. helps them to sustain survival and growth under iron-limiting conditions, which enhances their rhizosphere competence or ecological fitness, and simultaneously affects plant iron nutrition [116]. Differences in the number and composition of the amino acids present in the peptide chain of a particular siderophore are characteristics of each *Pseudomonas* species or strain that biosynthesizes it [117, 118]. These different siderophores can have large effects on the endophyte, as they can confer important selective advantages in iron-limiting conditions. Similar examples can be found on the expression of antibiotic biosynthesis [116, 119, 120], as well as on endophyte traits involved in triggering plant defense responses [101, 102].

Even though in its infancy, the development of functional gene markers at the endophyte level has started in bacteria contributing to soil-borne plant disease suppression. Genes and pathways in the biological control were identified by Park *et al.* [121] as well as sequence variations in functional genes associated with phenotypic variation at the subspecies level. Functional gene probes have been developed to rapidly identify bacteria of interest in the soil, such as for 2,4-diacetylphloroglucinol (2,4-DAPG)-producing *Pseudomonas* populations. Markers on *phlD* gene sequences (the key gene involved in the biosynthesis of 2,4-DAPG) have been used to quantify the abundance and to directly characterize the genotype of the most abundant *phlD*+ populations inhabiting the rhizosphere of various crops [122, 123].

The next step should then be, to our view, the generation and integration of this type of knowledge in plant-breeding strategies. Particularly, functional metagenomics started to explore the microbiome community but dissociated from their host, whereas in all the previously referred genomic approaches, the plant has been analyzed alone as a microbe-free organism. The time is ripe for an integrative approach such as functional hologenomics, where the functional diversity of endophytic microorganisms and the complex relations between them and with their host would be studied, together with the changes in the functional contributions of those communities along environmental gradients. This would enable to

manipulate endophyte communities improving plant fitness, either by introducing functional complementarities in the endophyte community that safeguard plant crop performance (e.g. productivity, stress tolerance/resistance) against unexpected environmental changes, or by introducing facilitator endophytes that increase the output of other species in the host microbiome. In extremis, it would allow for more sophisticated breeding processes, where new plant genotypes produced from *in vitro* cultures in form of callus or somatic embryos are already inoculated at this early stage with a fit-for-purpose-designed microbiome.

Improving crop host–endophyte interactions

The benefit obtained by improving endophyte traits to achieve desired agricultural performance on the holobiont depends not only on the endophyte population itself, but also on the environmental conditions (understood in a broad sense e.g. biochemical, physiological and cellular conditions of the host plant, root exudates composition in the soil, population and density of microbial community, abiotic factors) and on the responsiveness of the plant to its microbiome.

Therefore, when considering fitness and yield production of the holobiont, not only plant alleles or endophyte alleles conferring advantageous traits to the plant should be taken into account—and, being that the case, selected for—but also the responsiveness and the hosting ability of the plant to the association should be considered.

Variation in plant responsiveness to endophyte colonization can have large consequences in nutrient acquisition efficiency, in the potential to suppress plant pathogens and ultimately in plant fitness [124–127]. Host variation in responsiveness to beneficial microorganisms has been demonstrated in several plant–endophyte associations: significant genotypic variation in the responsiveness of legume cultivars to *Rhizobium* and of different crop species to mycorrhizal colonization has been observed, as well as variation on the capacity of different plant species to support root colonization by other endophytes such as *Trichoderma*, *Penicillium* and non-pathogenic *Fusarium* spp. (reviewed in [128]).

Furthermore, and at yet another level of interaction, plants seem to be able to differentiate between more- and less-cooperative endophyte partners and have the ability to sanction less cooperative strains (i.e. 'cheaters') through a nutrient embargo [129, 130]. In the reported examples, the most cooperative strains that transfer, for instance, more phosphorus or more nitrogen (in the case of arbuscular mycorrhizal fungi or *Rhizobium*, respectively) to the roots receive more carbon from the plant, and on the contrary, the gain in fitness of less-cooperative endophyte strains is reduced. Therefore, it seems that plants may have the power to discriminate between the best endophytes to harbor. Studies suggest that the capacity of the plant to sanction cheaters is a heritable trait and that this sanction ability (strength and sensitivity) may vary among plant genotypes, depending on the natural or artificial selection pressures to which their ancestors were exposed [131–133], and therefore, can be a selectable trait in plant-breeding programs.

Most breeding programs do not consider that the desired performance of a crop plant can be a function of an inherited ability of the host to interact with its associated microbiome [27]. Particularly, it is even likely that when selecting elite plants by traditional breeding, there has been some collateral selection for host–endophyte interaction [27, 78]. However, the strength of host–endophyte interactions could be improved in plant-breeding programs through new selection trajectories, where the sanction trait or the responsiveness to beneficial endophytes is considered as a major selection target i.e. where selection for enhanced plant performance is based on optimized interactions between host plants and their endophytes. Inoculation of individual plants or breeding lines with highly functional different species or isolates of bacteria/fungi would be the first step in any breeding program selecting for lines that are responsive to endophyte growth and health promotional effects [27].

Conclusions

Considering the holobiont and its hologenome as a unit of selection and improvement in plant breeding programs demands the development of new sophisticated approaches for the study of the association between a well-characterized holobiont genotype and phenotype. Nowadays, the new high-throughput genomic tools make this feasible, but there is still the need to integrate the ongoing efforts in different research areas into something like 'functional hologenomics' to identify genes, alleles and markers on the holobiont conferring agronomically interesting traits. Further, and ideally, this should expand into analyzing the symbiosis genes, genes for signal molecules, host–endophyte interaction signaling pathways and genes involved in metabolism/nutrient transport.

Key Points

- The perceivable crop 'plant phenotype' is the product of concerted and co-regulated expression of both crop plant and endophytic population's genes, together with environmental influences.
- Overlooking the contribution of endophytes to the overall phenotype (holobiont's phenotype) can hamper the development of reliable molecular markers.
- The availability of new molecular tools and technologies allows exploring and selecting for genetic variability resulting from crop-associated endophytes alone or in a consortium.
- Developing new molecular markers based on advantageous alleles or traits based on optimal plant responsiveness considering the endophytic associations will certainly increase the efficiency of plant-breeding programs for complex agronomic traits.

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