RTB Working Paper

Metabolomics in CGIAR Research Program on Roots, Tubers and Bananas (RTB)

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N O V E M B E R 2 0 1 9

CGIAR RESEARCH PROGRAM ON Roots, Tubers and Bananas

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Abstract

The CGIAR Research Program on Roots, Tubers and Bananas (RTB) is supporting investments in metabolomics in collaboration with the Fraser lab at Royal Holloway University London (RHUL). The metabolome is the global collection of all low molecular weight chemical compounds that are produced by cell metabolism in different sizes, polarity and quantities. It thus provides a direct functional readout of the cell's physiological status and activity under specific environmental settings. Metabolomics research in RTB has involved metabolite profiling for making significant marker-trait associations, assessment of genetic diversity and varietal identification. Therefore, this has included screening young plantlets as proxies for mature end-product quality (roots, tubers and banana fruits), identifying potential biomarkers for abiotic stress tolerance and for product-quality traits. In this paper, we review the main findings of this work, and discuss the implications for breeding programs in the RTB Program.

Acknowledgments

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Metabolomics in GGIAR Research Program on Roots, Tubers and Bananas (RTB)

METABOLOMICS: WHAT IS IT?

The metabolome, which metabolomics helps to identify and describe, is the global collection of all low molecular weight chemical compounds that are produced by cell metabolism in different sizes, polarity and quantities. It thus provides a direct functional readout of the cell's physiological status and activity under specific environmental settings. Moreover, the metabolome is the synthesis—the end product—of the various complex steps, including interactions, feedback loops and so on. These steps are controlled by the organism's genetics that take place in the cell (e.g., from DNA to RNA to protein, protein-protein interactions, enzyme activities, etc.) in response to stimuli, which eventually result in changes in the levels of many metabolites. Therefore, looking at this end product of metabolite changes both puts us closer to the trait of interest as well as leap-frogs over those other complex steps. Knowing and understanding the control of plant metabolism are not only critical for maximizing crop yield. More important, they allow us to address issues of human nutrition. Metabolites, however, can change in response to environmental factors (e.g., age of the plant, type of tissue, etc.), so the analysis is more complex and subject to confounding factors.

Translational metabolomics is the application of metabolomic techniques to improve crop yield and quality, going beyond biomarker discovery (Alseekh et al. 2018). In the CGIAR Research Program on Roots, Tubers and Bananas (RTB), investments have been made in metabolomics to set the base for translational metabolomics.

WHAT CAN METABOLOMICS GIVE US?

Metabolomics adds another set of extensive data that can be used to study and analyze complex living systems. It provides knowledge and clues of what large network of metabolic reactions (metabolic pathways) are involved in yield formation, attributes of product quality and tolerance mechanisms to biotic and abiotic stresses. Indeed, various studies show that agronomic and consumer traits are often directly associated with metabolite composition (Alseekh et al. 2018). Especially in combination with other "-omics" approaches, this can lead to the development of biomarkers associated with important traits that can be used in breeding programs, as well as identifying new and important targets for breeding. For example, work with tomato heirloom varieties showed large variability in metabolic quality traits, thus expanding options to breed for quality-related compounds in tomato fruits (Tieman et al. 2017). Likewise, the use of metabolomics to identify the lost diversity of such compounds in modern commercial tomato cultivars could allow us to reverse the genetic bottleneck created by domestication and breeding (Alseekh et al. 2018; Zhu et al. 2018). Therefore, by combining metabolomics with genomics and transcriptomics for breeding and trait elucidation, the -omics toolkit can:

- discover novel genetic variation to guide breeding decisions
- identify biomarkers associated with complex traits
- establish chemotyped core collections capturing biochemical diversity
- identify important targets for the breeding of complex traits

METABOLOMICS IN THE RTB PROGRAM

During its Phase 1 the RTB Program, led by Theme 1 (Nicolas Roux) and Theme 2 (Luis Augusto Becerra), supported investments in metabolomics in collaboration with the Fraser lab at Royal Holloway University London (RHUL), and initial work was directed at developing metabolite extraction protocols for the various RTB crops (RTB 2015). Thereafter, research has involved metabolite profiling for making significant marker-trait associations, assessment of genetic diversity and varietal identification. Therefore, metabolomics research has screened young plantlets as proxies for mature end-product quality (roots, tubers and banana fruits), and has attempted to identify biomarkers for abiotic stress tolerance and biomarkers for product-quality traits.

The collaboration with the Fraser lab has resulted in six publications to date, all in ISI (International Scientific Indexing) journals. Additional publications are in the pipeline, including a review of an RTB database of metabolites (Price et al. forthcoming). This latter publication also sets the scene for a chemical core collection complementing the germplasm genebanks and its use in the enhancement of genetic resources based on the crop's chemical composition. Thus, this can enhance the selection of parental lines displaying unique chemical features to confer resilience to climatic change, high physiological performance or the biosynthesis of high-value nutritional compounds for a healthy human diet.

RESULTS TO DATE

1. *Discover genetic variation to guide breeding decisions*

Metabolite profiling was carried out in RTB diversity panels of accessions, representative of the diversity of wild and cultivated varieties. By analyzing the metabolic profiles, we attempted to describe the genetic diversity in terms of discrete profiles. This resulted in sets of chemotypes for each RTB crop and also metabolic descriptors that allowed the different varieties of each species to be identified.

A panel of 38 banana accessions, representative of the diversity of wild and cultivated bananas, was analyzed to assess the range of chemotypes available globally (Drapal et al. 2019a). The 105 metabolites identified comprised a range of intermediates of primary and secondary metabolic pathways, with the widest metabolic diversity primarily found in the wild *Musa acuminata* and *M. balbisiana* accessions.

In yam, a diverse collection of 49 genotypes from five different *Dioscorea* spp (i.e., *D. rotundata, D. alata, D. cayenensis, D. bulbifera* and *D. dumetorum*) commonly used in yam breeding programs was selected. More than 200 compounds were routinely measured in tubers, providing a major advance for the chemotyping of this crop

(Price et al. 2017). The analysis of leaf and tuber material identified a subset of metabolites which allowed accurate species classification and highlighted the potential of predicting tuber composition from leaf profiles, based on the species classification. Therefore, *D. dumetorum* was defined by fatty acids, *D. rotundata* and *D. cayennensis* by TCA cycle intermediates and phosphate and *D. alata* and *D. bulbifera* both largely by sugars. This, however, needs to be validated using a larger number of samples and datasets.

Likewise, work in cassava has shown the value of metabolomics to classify accessions (Drapal et al. 2019b). More than 9,000 molecular features were detected in the untargeted analysis by liquid chromatography-mass spectrometry of *in vitro* plantlet samples. Targeted analysis datasets included more than 100 metabolites. The metabolite data analyzed were applied to describe the biochemical diversity available in the panel, identifying South American accessions as the most diverse compared with those grown today in Africa. Genotypes with distinct phenotypic traits showed a representative metabolite profile and could be clearly identified, even if the phenotypic trait was a root characteristic (e.g., high amylose content). Therefore, this illustrated the utility of the methodologies to chemically differentiate cassava accessions. This result was similar to that with yams, where by identifying the accession type by its leaf metabolite profile, the root characteristics could be inferred.

A recent publication on sweetpotato (Drapal et al. 2019d) assessed the metabolic diversity of 27 sweetpotato cultivars, including landraces and improved varieties. Researchers looked at metabolites both in leaves and storage roots and identified 130 metabolites. The results showed the value of metabolite profiling to breeding programs as a way to both identify differences in phenotypes/ chemotypes and characterize parental material for future pre-breeding resources. For example, the storage root data highlighted three cultivars which differed in their primary and secondary metabolite composition from most of the other accessions, and thus represent suitable parental material for breeding efforts.

The above studies have resulted in a collection of chemotypes for the different RTB crops (Figure 1) that constitute a valuable resource (Price et al. forthcoming). Allowing for species and, in some cases, accession differentiation according to their metabolic profiles, chemotyping is a means of (1) classifying diverse and redundant genotypes in over-populated genebanks, complementing and validating the use of genotyping approaches, and (2) utilizing the approach to precisely select parental materials used in future breeding efforts. In many of these studies, attempts were made to correlate metabolic profiles on young tissue (leaves or *in vitro* plantlets) to more mature plants in the field, and to the root or tuber products. Given the very different environments and the tissue types, it is not surprising that these correlations were not usually found. The metabolic profiles of the young tissue helped identify the accession or species; but only by knowing the particular root or tuber composition of that accession or species could inferences be made. This was not the result of direct correlations between leaf and root or tuber metabolites. Nevertheless, differences between species could be determined by the differing metabolic profiles in the young tissue, allowing for species identification in bananas, cassava and yam.

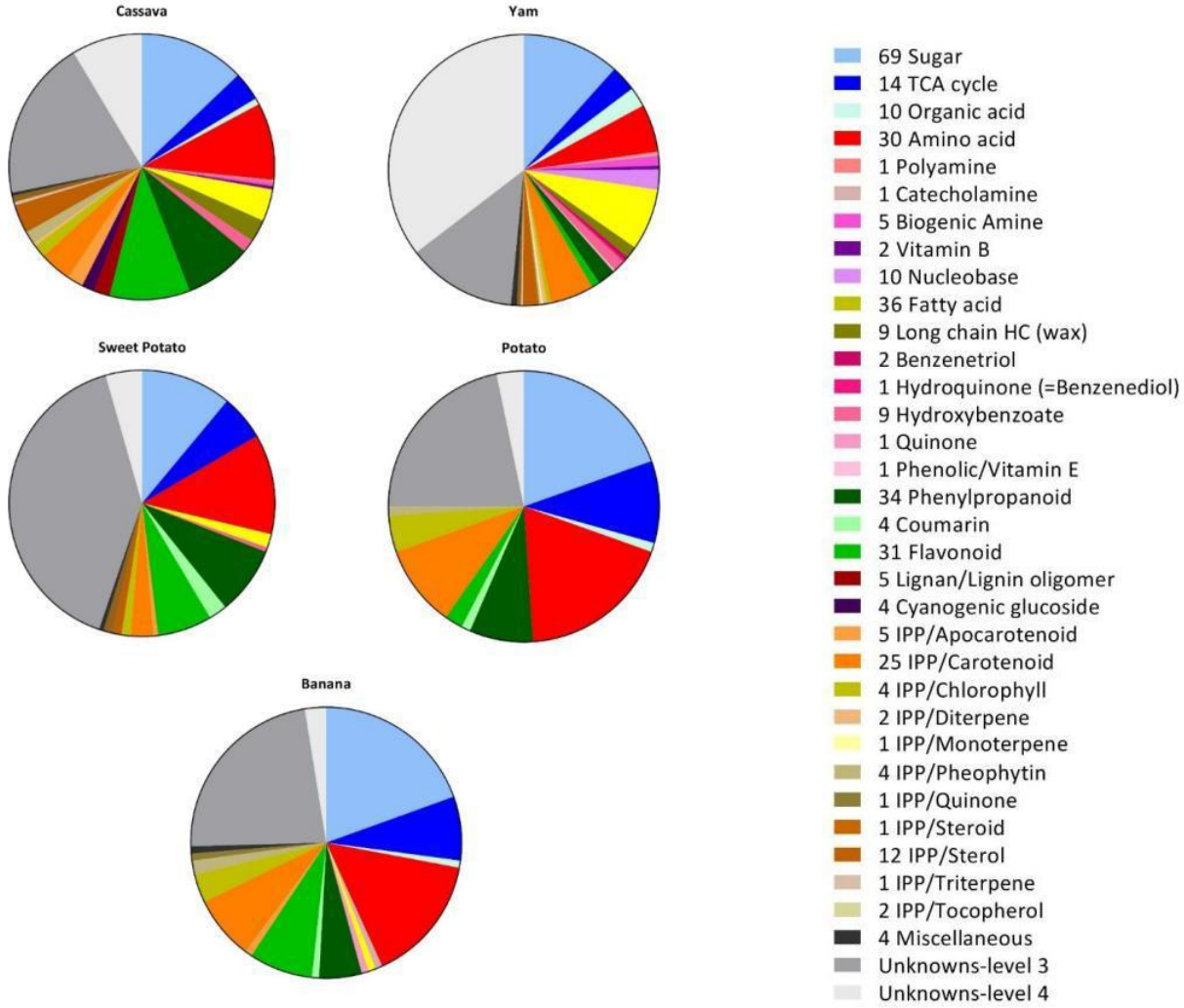

Figure 1. Metabolic profiles of chemical classes identified in RTB crops (From Price et al., Forthcoming)

2. *Biomarkers for biotic and abiotic stress*

Early work on potato attempted to use metabolomics to find biomarkers for drought tolerance (Drapal et al. 2017). Consequently, five varieties, differing in their drought tolerance, were grown under normal irrigation as control and under water-restricted regimen; metabolite profiles were developed. Results showed that nine metabolites in leaves changed in response to drought stress, suggesting probable pathways involved in the stress response. Previous studies have shown these metabolites to interfere with the ability of drought-tolerant plants to retain water from the soil or antioxidant mechanisms to protect from damage. However, no relationships were found between concentrations of these metabolites and the physiological measures for drought stress (i.e., relative water content, osmotic pressure or chlorophyll content). A confounding issue is that the lines did not differ sufficiently in their tolerance to drought, and it was therefore not possible to identify metabolites associated with tolerance mechanisms. Nevertheless, the study showed the value of such an approach. More

extensive drought-tolerance experiments need to be carried out in RTB crops, to be evaluated for both gene expression and metabolites. In addition, near-infrared spectroscopy (NIRS) calibrations were developed for each of the drought-responsive metabolites, so that inexpensive monitoring by NIRS in large numbers of genotypes can be carried out for exploration of their functional role and for genetic studies.

Work in cassava has shown the value of metabolomics to identify probable metabolites that could be involved in biotic stress resistance mechanisms (Drapal et al. 2019b). A line susceptible to thrips had higher free catechin/epicatechin levels, which could indicate lower condensed tannin levels thought to provide resistance to the pest. Likewise, additional metabolic profiles conducted on cassava whitefly-resistant families of accessions highlight the importance of structuring families with contrasting traits under similar genetic backgrounds (Becerra, personal communication), thus facilitating the discovery of biomarkers for breeding. In banana, metabolic profiling showed several metabolites to be more prevalent in the B genome, which could be related to biotic stress tolerance (Drapal et al. 2019a). For example, the metabolite data of Calcutta 4, a parent usually used in crosses to confer resistance to various diseases, showed above-average levels of rutin, chlorogenic acid and caffeoyl-malate, which could be related to its resistance traits. Future metabolite analysis of pulp and peel will show whether these metabolite differences can also be detected in the consumed banana product(s) of offspring and influence the quality of banana pulp.

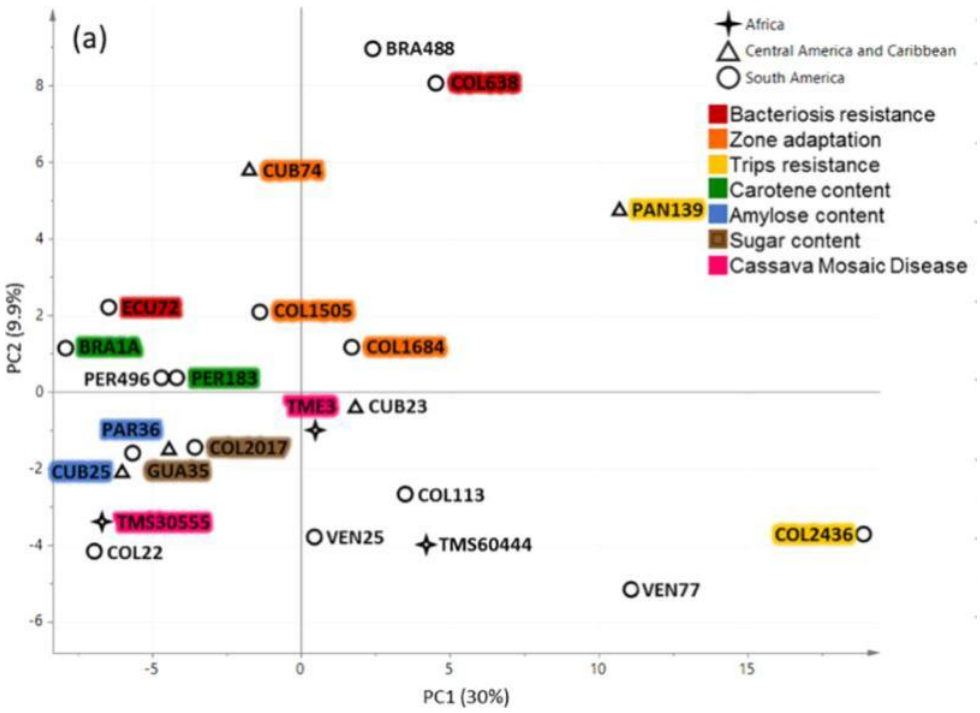

Figure 2. PCA of *in vitro* plantlets of 23 cassava varieties based on metabolites identified. Score plot of varieties includes additional information about region of origin and characteristic traits (from Drapal et al. 2019b).

3. *biomarkers for product quality and nutritional status after processing and cooking*

Metabolites are directly related to flavor and aroma of food products. They are also involved in carbon partitioning into yield components, and the eventual nutritional composition of the product, such as starch structure, sugars, antioxidants and carotenoids.

Sensory evaluations were carried out in a panel of potato from four germplasm groups (i.e., advanced breeding lines, diploid landraces, wild potatoes and diploid biofortified-bred clones) in fresh tubers and boiled tubers after 4 months of storage. These were followed by metabolite profiling, to identify metabolites associated with potato flavor components that may be included in breeding programs. The sensory survey addressed various quality parameters such as potato flavor intensity, sweetness, savoriness, sourness, bitterness and mealiness. The profiling identified 77 metabolites and allowed the differentiation of different germplasm groups (Drapal et al. 2019c). They also showed why certain wild potatoes were preferred during domestication. One of the group-specific properties was no significant change of carotenoid levels after cooking in the native hybrids bred for high carotenoid content. Although group-specific metabolite compositions were maintained upon cooking, the cooking process affected a broad range of metabolites. Glycoalkaloid levels decreased significantly after cooking, but less so in wild species. Breeding lines showed less starch degradation and resulting release of sugars, consistent with this being an important quality trait in breeding programs. The associations between metabolites and sensory properties are still being analyzed and will result in an additional publication. Consequently, this work provides guidelines for what metabolites to screen for after harvest, cold storage and cooking for product quality. The sensory associations should provide breeding targets for breeders.

Work on sweetpotato (Drapal et al. 2019d) assessed the metabolic diversity of 27 sweetpotato cultivars including landraces and improved varieties. It also looked at carotenoid content, which is a very important target in sweetpotato-breeding programs as a biofortication strategy to combat vitamin A deficiency (Low et al. 2017). The use of metabolomic protocols provided a more exact profile of the different carotenoids present in roots—much more than commonly used spectrophotometric approaches. For instance, significant levels were found of mutachrome, a carotenoid similar to β-carotene but with less pro-vitamin A activity. Consequently, this work suggests that spectrophotometric approaches are good for screening large amounts of samples, yet require a validation step with more refined protocols for the more promising lines. This was further confirmed in follow-up work by Drapal and Fraser (forthcoming).

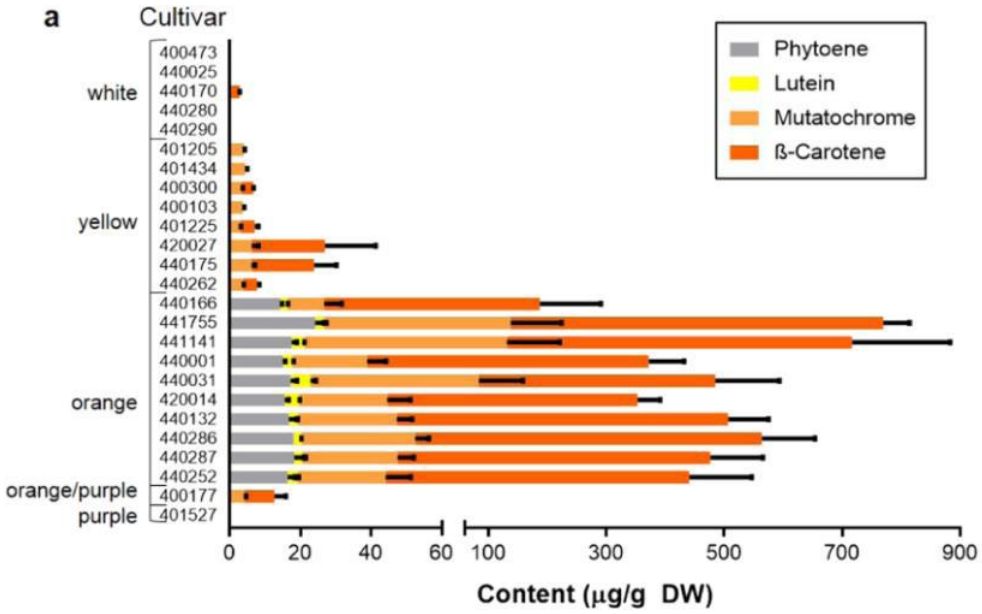

Figure 3. Carotenoid profiles of sweetpotato storage roots as µg/g DW (dry weight). Cultivars were grouped into phenotypes by storage root pigmentation (from Drapal et al. 2019d).

In cassava, each trait could be identified by its specific metabolite composition (Drapal et al. 2019b). For example, the varieties with a higher amylose root content were correlated with higher levels of TCA cycle intermediates in the leaves. As a result, the variety with high culinary quality correlated with higher levels of monosaccharides and intermediates of the TCA cycle. This suggests increased levels of glucose and fructose for transport to starch biosynthesis in the roots as previously observed, and so leads to the hypothesis that *in vitro* leaves can be used to screen for root phenotypes. In 2020 RTB support on metabolomics will focus on the global cassava genome-wide association study (GWAS) population, developed by CIAT, to validate the power of biomarker screening in leaves tissues as young as those from *in vitro* plants.

In yam, further work (Price et al. 2018) looked at the carotenoid profiles in the diverse yam collection; several carotenoids were identified. What is more, color of the tuber flesh was not necessarily indicative of pro-vitamin A activity. The carotenoid epoxide mutachrome was present in various accessions, and it was also identified in various sweetpotato accessions. As it has less pro-vitamin A activity than β-carotene, this must be taken into account when selecting for high carotenoid lines. Nevertheless, where levels are relatively high in yam, the mutachrome can contribute as much pro-vitamin A activity as can yellow cassava. Another very interesting carotenoid derivative, C25-epoxy-apocarotenoid persicaxanthin, has potential implications for tuber dormancy. It can be indicative, indirectly, of ABA levels (Schwartz et al. 2003) and thus could be a breeding target. As for sweetpotato (Drapal and Fraser, forthcoming), the metabolic profile of carotenoids in the yam diversity panel showed that tuber color is not good enough to determine pro-vitamin A activity. Even ultra-performance liquid chromatography, just as in sweetpotato, does not discriminate well enough between carotenoids due to the low levels and enrichment required.

4. Challenges and opportunities

The RTB Program's support of metabolomics work has yielded valuable new information: a set of chemotypes for all the crops (Price et al. forthcoming). By developing metabolic profiles on diversity panels put together by RTB scientists and breeders, important targets for breeding are being elucidated. These include particular metabolites that may be linked to traits of interest and accessions and breeding lines that can be the source of important metabolites for incorporation into breeding programs.

The work to date provides evidence that metabolic profiles can be used to differentiate between species, and this can be used to help manage genetic resources. We note, however, that other approaches such as DNA fingerprinting can be applied more easily but presents serious limitations when trying to unravel multi-allelic variation. Metabolite profiles, on the other hand, do not have this limitation. Thus, metabolic profiling can help to fill some gaps or uncertainties when needed and provide a more comprehensive picture.

Future analysis using bigger datasets will be needed to validate whether leaf metabolic profiles can be representative or even predictive of storage root or tuber metabolite composition. If so, initial phenotypic screening of breeding programs could be undertaken on leaf material. Too, profiling of root and tuber crops would be significantly more rapid and conducted prior to tuber formation, thus requiring less labor for material harvesting and rendering analyses cheaper and easier.

Metabolomics requires advanced lab equipment and highly trained technical staff as well as a highly complex analysis of results. Therefore, it is not likely to become a component of breeding teams. Rather the expertise can be accessed via collaborations, as has been done between RTB Program scientists and breeders with the RHUL Fraser team. This said, the analytical platforms are becoming more competitive in price and easier to use. In addition, numerous low- and medium-income countries are realizing the need for a national center that can provide metabolite-profiling resources as a tool in food analysis/ safety, quality evaluation and support across crop breeding programs. The RHUL lab has also run several metabolite training schools for early-stage researchers. It is hoped that in the near future the RHUL group will be able to run one of these activities in a target country such as Tanzania.

In any case, identifying biochemical signatures of phenotypic traits would allow metabolite marker-based breeding. Since metabolic profiles can provide a direct biochemical measurement of quality traits, they can be utilized as markers to investigate trait inheritance. Thus, NIRS equations can be developed for metabolites that are associated with particular traits, making the screening more high-throughput (Tumwegamire et al. 2011). Combining genomic analysis with metabolomics is leading to the use of metabolic GWAS (mGWAS), in which metabolites and their levels are treated as a trait and associated molecular markers identified. This has proven very powerful to elucidate the metabolites involved in tomato flavor and aroma, while developing molecular markers to incorporate them into breeding programs for fruit quality (Tieman et al. 2017; Zhu et al. 2018). In addition, it has the potential to assist in the identification of metabolites. In 2020 the global cassava GWAS

population will be used to undertake an mGWAS in order to identify important unknown metabolites associated with tolerance to short shelf life, high β-carotenoid potential and soft texture after cooking. All these traits have a huge influence on consumer acceptance/preference.

A recent biofortification study revealed phenolic compounds as inhibitors of iron absorption by the human body (Andre et al. 2015). Therefore, the metabolite data developed for the biofortified potato lines (Drapal et al. 2019c) present an ideal resource to identify potatoes suitable for such biofortification efforts. And though this needs further validation, it can help identify additional breeding targets that need to be pursued together with iron content.

Consequently, when performing comparative analyses of crop growth under different environments, quantifying the contributions of biochemical signatures toward phenotype is often simpler than for genetic markers, especially in highly heterozygous crops like RTBs. This gives rise to the potential to generate chemotype core collections for use in breeding. With this approach material selection is based on the fixing of a complement of biochemical signatures that confer desired characteristics more robust to environmental variation. This is contrary to genotypic core collections whereby breeding tries to fix gene variants that can then often harbor different traits under different environments.

Metabolomics can thus enhance breeding programs in various ways. It helps describe and differentiate genetic diversity from which new breeding lines can be developed. It can identify novel breeding targets as metabolic pathways involved in trait formation are identified, and these targets can then be assayed either chemically, or converted into more high throughput assays such as NIRS, and/or molecular markers and new chemical functions identified through mGWAS assays. In the case of NIRS, this spectrophotometric technique can be used to screen large numbers of early selection material. The most promising genotypes are then validated by more precise assays, such as when breeding for high β-carotene content, in the presence of other carotenoids that contribute to flesh color but might not have high pro-vitamin A activity.

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RESEARCH PROGRAM ON Roots, Tubers and Bananas

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