Chromium interactions in plants: current status and future strategies†

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Chromium has received relatively little attention from plant scientists compared to other heavy metals in recent times in spite of it being a very a hazardous environmental pollutant. One of the reasons for this is the complexity of the metal's interactions with biological systems and the difficulty in studying them. Although the possible mode of entry into the plants, resultant toxicity mechanisms and tolerance potential has been worked out in plants there is still a need to get a complete picture of the Cr–plant interactome. With the advent of hyphenated technologies and global gene/protein and metabolite expression/quantification techniques, studies to elucidate the complete metallome are possible albeit resource intensive. This minireview focuses on the recent developments in the field of Cr–plant interactions and proposes a model using a systems biology and integrated -omics approach to decipher the intricacies of Cr–plant interaction.

1. Introduction

The extensive use of various chromium compounds in dyeing, tanning, steel plating and other metallurgical industries has caused increasing concern about environmental contamination with this element. On the other hand it is also known that chromium can be both beneficial and toxic to animals and humans depending on its oxidation state and concentration. At low concentrations, trivalent Cr is essential for animal health,[1] whereas hexavalent Cr is a potent, extremely toxic carcinogen and may cause death to animals and humans if ingested in large doses.[2] Ingestion of plant-derived Cr-containing food materials, primarily food crops, provides a major portion of the daily Cr intake. It is therefore crucial to determine which forms of Cr are present in plant tissues, in what way they interact with metabolic and physiological processes and the magnitude of their accumulation in plant parts. Over the past decades, scientific attention in heavy metal studies has moved from total element determination towards speciation analysis. This is all the more important in metals like chromium which exist in two or more electronic or oxidation states *in vivo* because of the diverse nature of biological interactions like toxicity, mobility, bioavailability, and bioaccumulation these states evoke.[3] In recent times, metallomics has been introduced as an all-inclusive extensive concept, in which the role of all metals/metalloids in a given system is considered in its entirety.

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The challenge facing systems biology and metallomics is the integration of proteomic, transcriptomic, and metabolomic information to give a more complete picture of the metal's involvement in molecular, cellular and organismal processes. Metallomics focuses on the entirety of metal and metalloid species as opposed to metalloproteomics, which is focused on the functional exploration of metal binding proteins and metalloproteins (these two are mainly differentiated by the degree of affinity and endurance of interaction[4]). The complete metallomic picture of an organism would therefore involve not only the metal-associated biomolecules but also the dynamics of these and other biomolecules as an effect of metal intrusion. This essentially will involve bridging of experimental data from the lower order -omics platform namely metallo-transcriptomics and higher order -omics platform – metallo-metabolomics with metalloproteomics. The metallotranscriptome can be ideally defined as the map of the entire transcriptome in the presence of biologically or environmentally relevant concentrations of an essential or toxic metal, respectively. Deciphering a metallome, according to Szpunar,[5] should inform us of among other things its coordination environment, into which biomolecule it is incorporated or by which bioligand it is complexed. In addition to this, chemical perturbations (especially in the nucleic acids) brought about by the metal which could bring about drastic phenotypic manifestations should also be included in the fabric of the metallomics picture. The importance of the metallo-transcriptome is further emphasised because of the ability, especially of the toxic species, of metals to act as epigenetic factors involved in DNA and histone modifications.[6] In similar terms, the metallometabolome would constitute the complete pool of small metabolites in a cell at any given time and this would give rise to the whole metallointeractome and knowledge of this would be of paramount importance in comparative metallomics dealing with toxicity and drug discovery. The complete picture of a metallome as discussed above would still be incomplete without the temporal and spatial localization of the free metal species within the cell and its organelles. In case of oxyanions like Cr the task of constructing the metallome is made no simpler due to the fact that not only does Cr change its oxidation state from Cr(III) to Cr(VI) and back depending on the protonic environment but also because of the analytical sophistication required to detect these changes. In this article we cover current strides that have been made in the areas of chromium–plant interaction in terms of its speciation dynamics, metabolic implications and its potential biological significance and possible methodology to study the Cr metabolome in plants as a whole.

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2. Current status of chromium–plant interactions

Cr is toxic to plants and is a non essential element. The degree of toxicity differs according to the oxidation state of Cr with Cr(VI) being more toxic than Cr(III),[7] making it one of few elements that exhibits different physiological and toxicological effects depending on its oxidation state. Cr interacts with plants at a cellular level mainly by producing reactive oxygen species such as superoxide radicals, hydroxyl radicals and hydrogen peroxide either by direct electron transfer involving metal cations, or as a consequence of metal-mediated inhibition of metabolic reactions.[8] Considerable strides have been made in the area of plant metallomics particularly with reference to toxic metals like Cd, As and Al[9–15] and essential metals like Zn and Cu,[16–18] comparable to that of animal systems. Chromium has been extensively studied in animals and a considerable body of knowledge has been accumulated with regard to the nature of its toxicity and essentiality. On the other hand, Cr–plant interactions remain poorly understood, because of the paucity of precise and accurate analytical methods rather than the lack of experimental endeavours. The reason for this is the degree of sophistication involved in the determination of the oxidation state of Cr *in vivo*. Another reason to a lesser extent is the relative importance of the metal *per se* in plant systems, unlike animal systems wherein Cr is a potential carcinogen at one end of the scale and a possible dietary supplement at the other end of the scale. The interest in the Cr metallome for all practical considerations would be driven by the following paradoxical goals: (i) to reduce its uptake in crops so that it does not affect growth and yield and at the same time regulate minimal uptake for the production of naturally fortified dietary supplements; (ii) to increase uptake in hyper accumulators and keep toxicity to a minimum for the completion of its lifecycle.

2.1 Speciation dynamics

The micro-proton-induced X-ray emission (μ-PIXE) technique is an effective way to quantitatively determine the amount of Cr in plants accurately and precisely[19,20] although knowledge of speciation can be gained by X-ray absorption near-edge structure (XANES), X-ray absorption spectroscopy (XAS), anion-exchange HPLC–ICP-MS and ion-pairing HPLC with diode-array and ICP-MS detection.[21] Cr(VI) is actively taken up in a metabolically driven process in contrast to Cr(III) which is passively taken up and retained by cation exchange sites of the cell wall.[22,23] The pioneering X-ray absorption near edge structure (XANES) study[24] on Cr speciation

interconversion showed that Cr(VI) is converted to Cr (III) by plants in the roots. The root to shoot translocation of Cr was extremely limited because of the propensity of Cr(III) to bind to cell walls, thus roots exhibit a 100 fold higher Cr accumulation than shoots, regardless of the Cr species supplied. Extended X-ray absorption fine structure (EXAFS) was used by Sawalha *et al*. to provide information regarding the coordination environment and the nearest neighbouring atoms and the ligands involved in Cr binding by saltbush biomass.[25] In addition, XANES was used to provide information about possible changes in the oxidation of Cr atoms bound to the biomass. The esterified and hydrolysed saltbush biomass was subjected to varying pH profiles and it was found that binding of Cr(III) and Cr(VI) to stem, leaf and flowers varied with the protonic environment, in addition the relative number of carboxyl functional groups on the biomass was found to influence binding. The results of the XANES analysis showed that samples were in an octahedral arrangement of oxygen atoms around the central Cr(III) atom. Bluskov *et al*.[26] reported that in plant tissues, Cr (III) was detected, primarily as acetate in the roots and oxalate in the leaves. X-Ray microprobe showed the sites of Cr localization, and probably sequestration, in epidermal and cortical cells in the roots and epidermal and spongy mesophyll cells in the leaves.

2.2 Metabolism and oxidative stress

The complete metabolic picture of antioxidant pathway and related metabolites[8] shows that reduced glutathione (GSH) acts as a signal intermediate in increasing the free radical scavenging enzymes such as ascorbate peroxidase under Cr(VI) stress in addition to a differential response to ascorbic acid signalling by Cr(III) and Cr(VI) resulting in different manifestations of toxicity. The reduced rate of GSH/GSSG ratio decline under Cr(VI) increases metabolic load to maintain a minimum redox buffer status of the cells. On the other hand, in the case of Cr(III) stress, a sufficient amount of ascorbic acid was enough to counter oxidative stress. Recently Pandey *et al*. found that NADPH-dependent superoxide production in pea root plasma membrane vesicles damages pea root plasma membrane structure and function, resulting in decreased photosynthesis and poor plant growth.[27] Cysteine, which generally occurs in many oxidation states with sulfur *in vivo* and by virtue of its ability to affect structural stabilization, catalysis, redox-activity, and metal-binding, is known to increase and confer tolerance to plants under Cr stress.[28] Cr(VI) has also been shown to impair nitrogen metabolism due to reduction in the activity of nitrate reductase, nitrite reductase, glutamine synthetase, glutamate dehydrogenase and urease.[29] Cr and its role in photosynthesis has been widely studied[30,31] and a clear picture of its interaction with plants has since emerged. Cr(VI) did not affect the Fv/Fm ratio of chlorophyll fluorescence implying that the primary photochemical processes in PS II were not affected. On the hand, excitation capture by open PS II centres, *in vivo* quantum yield of PS II photochemistry, and electron transport rate were significantly reduced by Cr(VI). The coefficient of photochemical quenching was reduced with a concomitant increase in the coefficient of non-photochemical quenching. This suggests reduced demand for ATP and NADPH due to inhibition of CO_2 assimilation, supporting the view that disorganization of the chloroplast ultra structure and inhibition of electron transport processes due to Cr and a diversion of electrons from the electron-donating side of PS I to Cr(VI) is a possible explanation for Cr-induced decrease in photosynthetic rate. DNA and Cr interactions have been less studied in plants. Nonetheless, a study by immunolabelling using a monoclonal antibody against 5-methylcytosine and methylation-sensitive amplified polymorphism (MSAP) showed cytosine-hypermethylation and extensive methylation changes in CCGG-sequences.[32] The mechanism of transport of Cr into plants has long eluded researchers. It was hypothesised that Cr competed with transporters of essential elements that are similar in electronic structure to gain entry into the plant system.[33] Very recently it was found that a significant decrease of sulfate uptake rates observed in Cr-treated plants was accompanied by repression of the root low-affinity sulfate transporter (BjST1), suggesting that the transport of chromate may involve sulfate carriers. Once absorbed, chromate induced genes involved in sulfate assimilation (ATP-sulfurylase: *atps6*; APS-reductase: *apsr2*; Glutathione synthethase: *gsh2*) and the accumulation of cysteine and glutathione, which may suggest that these reduced S compounds play a role in Cr–plant interactions.[34] A proteomics study by Labra *et al*. showed that proteins induced by Cr exposure are principally involved in oxidative stress tolerance or in other stress pathways.[35] Induction of proteins implicated in sugar metabolism was also observed. As far as we know this is the only study on Cr-induced proteomic changes in plants. cDNA-AFLP markers have been used to identify candidate genes involved in the regulation of the response to chromium,[36] results were validated by semi-quantitative RT-PCR analysis of seven candidate genes. Interestingly, it was found that there exist common mechanisms of gene regulation in response to Cr, pathogen attack and senescence-mediated programmed cell death, suggesting a role for the genes isolated in the cross-talk of the signalling pathways governing adaptation to biotic and abiotic stresses.

2.3 Chromate reductase activity in plants

An assortment of bacteria isolated from a diverse range of environments have been recognized to possess Cr(VI)-reducing properties. Opperman and Heerden have recently purified and characterized a membrane-associated chromate reductase from *Thermus scotoductus*.[37] In contrast, no enzyme is known to catalyze Cr(VI) to Cr(III) in plants although there is evidence that reduction of Cr(VI) does take place in the plant cell. This prompted us to conduct a thorough amino acid sequence comparison of the above characterized enzyme with all available plant genome and plant protein databases (NCBI Plant blast and PlantGDB blast). Surprisingly an FAD binding dihydrolipoyl dehydrogenase/oxidoreductase (LPD2) produced a significant 45 percent similarity of sequence in various plants with 60 percent positives and a low gap of 2 percent. Dihydrolipoamide dehydrogenases belong to the family of pyridine nucleotide-disulfide oxidoreductases

(class I active site), which also includes glutathione reductase, and are identifiable by the consensus patterns around the two redox-active cysteine residues located near the *N*-terminus. A homology model was built (Fig. 1) with the protein sequence of FAD binding/dihydrolipoyl dehydrogenase/oxidoreductase (LPD2) of *Arabidopsis thaliana* (highest blast hit). BLASTP2 was done with the ExNRL-3D database; sequences of known structure were scanned for similarities to target. SIM alignment was done and all templates with sequence identities above 25% were selected and a model was generated from an exPDB database scan by the ProMod II method.[38] Missing side chains and deleted loops were added and energy minimization was done after adding hydrogen atoms. The model was verified using Anolea, Gromos and Verify3D. The model has three nucleotide binding sites, a proton accepter at residue 363 and a disulfide bond between residues 45 and 50 which is redox active. The enzyme operates between glycolysis and the citric acid cycle, but there is a possibility that it could have additional physiological functions. Catalyzing the reduction of Cr(VI) could prove advantageous in detoxification. Hence it is possible that in the presence of an electron donor (NADP/NADPH) the enzyme LPD2 could carry out the seemingly physiologically unrelated function of chromate reduction in plants. This is quite possible since plant NAD(P)H:quinone oxidoreductase (NQR), which is a functional homolog of animal DT-diaphorase that has shown

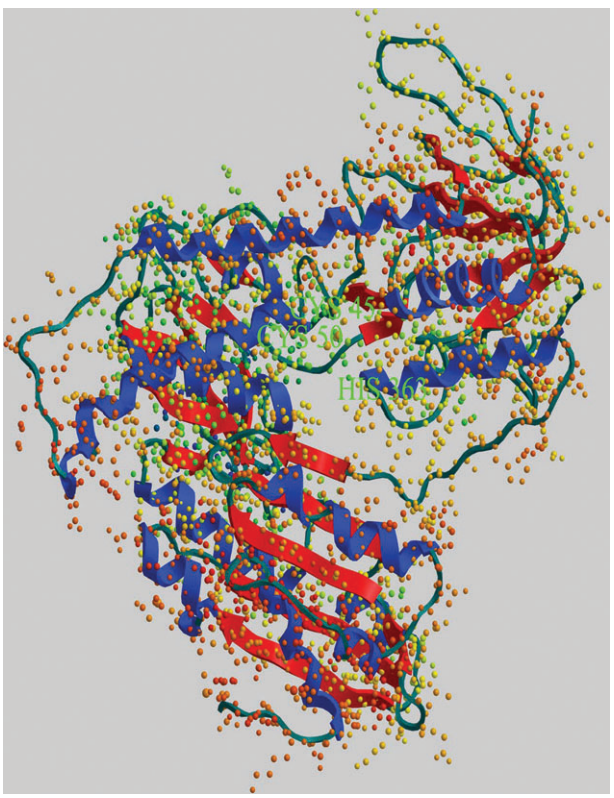

Fig. 1 A homology model (refer to the text in section 2.3 for details) of FAD binding dihydrolipoyl dehydrogenase/oxidoreductase (LPD2) of *Arabidopsis thaliana* exhibiting 46% sequence similarity with protein exhibiting chromate reductase activity in *Thermus scotoductus*. The model suggests a theoretical possibility that LPD2 could reduce Cr in plants, which is yet to be experimentally verified.

33–38% (7% less than LPD2) sequence identity to prokaryotic chromate reductases, exhibited detectable chromate reductase activity in *Arabidopsis thaliana*.[39] It should be noted here that the above model is purely suggestive and theoretical, and as of now there is no experimental verification of the enzyme LPD2's possible capabilities to reduce Cr in plants.

3. Experimental and analytical methods for Cr–plant interaction studies

A continuing shift towards hyphenation in analytical methods and the integration of biological experimentation and bioinformatics with these methods has thrown up an immense amount of meaningful data towards understanding the complete metallome of various trace elements. An all-inclusive metallomics study of Cr would involve transcript profiling for gene expression, global DNA methylation detection, proteomic characterization by online coupling of electrophoretic techniques, chromatographic separation techniques, targeted metabolite analysis and a high-power sensitive and element-specific oxidation state detection system seamlessly integrated with algorithmic data analysis. The initial step in wholesome metallomics would be to start with the gene-driven approach – to ask the question: what is the transcript profile of plants under several different environmentally and biologically relevant concentrations of Cr(III) and Cr(VI)? The application of microarrays for gene expression profiling has been demonstrated to be one of the most powerful and direct ways of using the sequence data for functional studies. It represents an approach that is both comprehensive in its scope and high-throughput in its application and can be effectively applied for deciphering the transcriptome in the case of Cr and, in addition, on-lining it with the other -omics and hyphenated analytical methods would be a good strategy. In this hypothetical case we take the Indian mustard (*Brassica juncea*) as the paradoxes in objectives (Fig. 2) are essentially satisfied because of the fact that it is an oilseed crop and also a known hyperaccumulator[40] of Cr and also because the whole genome is due shortly.

3.1 Transcript profiling

Transcript profiling can be done by taking the Whole Genome Array (WGA) as against cDNA arrays as they often miss very low abundance and non-polyadenylated transcripts and are often devoid of transcripts that are expressed in response to a specific physiological or environmental condition.[41] WGA tiling arrays can also detect alternatively spliced forms which may not have been previously known or predicted. These arrays can be used for gene expression studies by hybridizing targets made from RNA samples of different tissues *viz*., flower, leaf, root, stem cultured cells, all exposed to different concentrations of Cr(III) and Cr(VI) in medium (Fig. 3). Total RNA is isolated from these samples and double strand cDNA is synthesized, it is used as a template for transcription of complementary RNA (cRNA) which equally represents all expressed gene products in the total RNA, in addition to serving as amplification of targets in adequate magnitude for hybridization to WGAs. After hybridization signal detection and data processing is carried out. The normalized signal

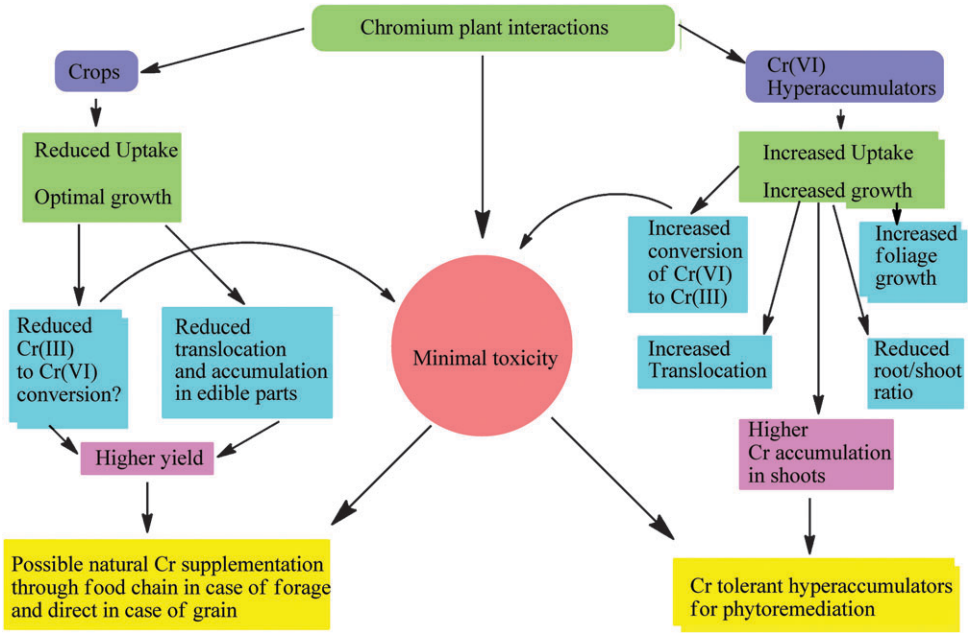

Fig. 2 Schematic representation of the Cr–plant relationship. The twin goals are in conflict with each other wherein one is to evolve tolerant crops that take up less Cr and grow well in Cr contaminated soils and the other is to evolve efficient hyperaccumulators that take up high amounts of Cr and clean up the environment.

intensities, devoid of noise, of each target from repetitive hybridizations is averaged and change under treatment conditions is calculated as the ratio of the average intensity in the treated samples to that in the appropriate control sample.[42] Simultaneously, WGA can also be used to map sites of DNA methylation (also known as the methylome) within the Brassica genome. Zilberman *et al.* have perfected this technique in *Arabidopsis thaliana*. The simplified procedure here is to use an antibody that recognizes methylated cytosine bases of genomic DNA of flower, leaf, root, stem and cultured cells all exposed to different concentration of Cr(III) and Cr(VI) in medium. These regions are immunoprecipated then these DNA fragments are super amplified to get higher DNA yield and later they are cut down to small DNA fragments (to increase hybridization efficiency) and hybridized with the WGA.[43] A similar bioinformatics analysis of this microarray can be done to obtain expression patterns. Microarray data of these two processes should be superimposed to obtain a map which would include epigenetic aspects of Cr treatment. Alternatively, total DNA of the samples can be isolated and digested and a global DNA methylation pattern for quantification of 5-methyl-20-deoxycytidine (5-mdC) is arrived at by isocratic cation exchange high-performance liquid chromatography according to Rozhon *et al.* and this can be compared with the processed WGA data.[44] This would be an important aspect in the metallome study as dose-related increases in sequence alterations, extensive methylation changes in CCGG-sequences, and genome-wide hypermethylation leading to epigenetic silencing or reactivation of gene expression has been reported due to Cr.[45] The transcriptome analysis is likely to show functionally undefined hypothetical genes and genes with annotated functions as affected by Cr.

3.2 Proteome analysis and elemental quantification

The next step would be a high-throughput proteomic method, based on LA-ICP-MS to detect Cr-proteins in protein bands of 1D gel electrophoresis (1D-GE) or protein spots separated after 2D gel electrophoresis (2D-GE) and matrix-assisted laser desorption/ionization with time-of-flight mass spectrometry (MALDI-TOFMS) analysis of tryptic 2D electrophoresis (2-DE), spot digest and peptide matching with the *Brassica juncea* database. The transcriptome and the proteome data are superimposed to assess the parallelism between DNA transcription and protein expression (Fig. 4). The proportion of detectable proteins to that of the transcriptionally active genes will throw light on the mechanism of action of Cr in plants in detail. In addition to protein profiling, protein–DNA interaction is of importance especially in metal studies because chromium complexes are known bind to DNA, causing lesions that can alter interactions with proteins and disrupt normal cellular function. Very recently Stansfield *et al.* have improvised protein microarrays to study protein–DNA interaction.[46] They used an array of various proteins created on a nitrocellulose membrane and screened it by using labelled chromium-modified DNA probes containing appropriate promoter regions, this method has been used to discover DNA binding ability in proteins with other identified functions. This method offers a high-throughput means for recognizing proteins that bind to a particular DNA recognition sequence, an achievement that is hard to accomplish using other methods. The logical extension of this would be to conduct crystallographic studies of the induced proteins. Single crystal microspectrophotometry has also been used on synchrotron X-ray beamlines to enable *in situ* observations of the chemical states of metals during crystallographic

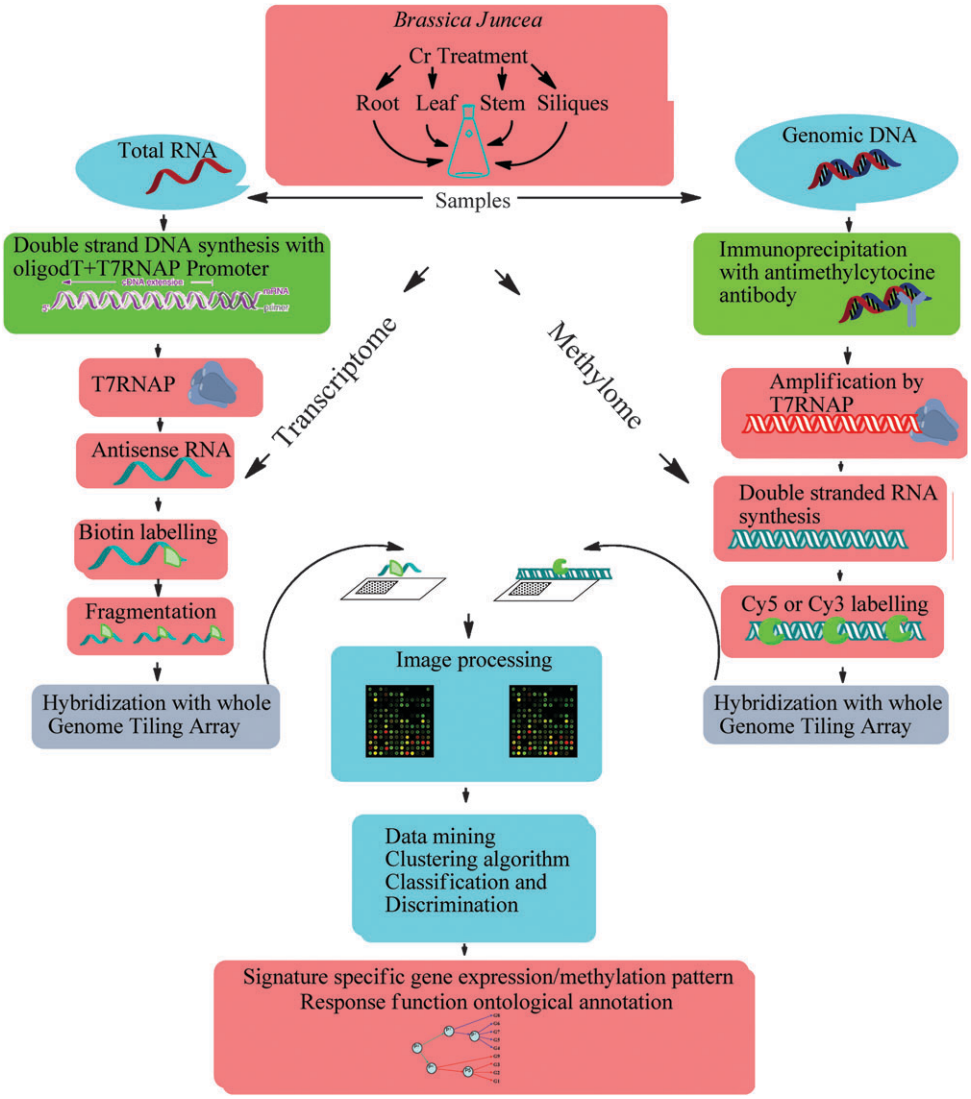

Fig. 3 Schematic representation of a proposed strategic approach for analysis of the transcriptome and methylome exemplified for a hypothetical experiment with Indian mustard under different Cr(III) and Cr(VI) concentration levels.

experiments.[47] Alternatively XANES at the Cr K edge has been used successfully to determine Cr oxidation states in wood and plant tissues.[48,49] A combination of X-ray synchrotron radiation microbeams and nuclear microprobe for quantitative chromium and chromium oxidation state mapping in cells can be used, which is described by Ortega *et al.*[50,51] μ-PIXE sample preparation for determination of metals in plant tissues has been standardized.[19,20] The freeze-drying technique, which involves ensured good preservation of the cellular structure and satisfactory lateral and spatial resolution, enables true elemental imaging and quantification of elements at a cellular level. Tissue samples are excised, sectioned and cryofixed in liquid nitrogen on a time scale of less than 5 seconds and freeze dried for 48 h and packed in 2% ethylene dichloride in target holders and stored in desiccators.

3.3 Metabolomic studies

The next step in the strategy is to construct a complete metabolic profile. The components of the metabolome can be seen as the final products of gene expression and describe the biochemical phenotype of a cell or tissue in comparison with the molecular biological genotype. Quantitative and qualitative measurements of all cellular metabolites consequently provide a clear insight into the biochemical status of an organism, an extension of proteomic expression data in relation to pathway dynamics that can be used to monitor and assess gene function.[52] The proven suitable method for a complete metabolite analysis in the case of metals is liquid chromatography coupled to mass spectrometry.[53] The procedure would involve an LCQ-Duo ion trap mass spectrometer fitted with an electrospray source. This hyphenated mass spectrometry method would offer good sensitivity and selectivity, but relatively longer analysis times (Fig. 4). The analysis of the metabolome would provide the most complete functional relationship between Cr and plants. On the other hand, transcriptome and proteome profiles can effectively point to functionality, and consequently a judicious integrated approach can be adopted with available resources. The

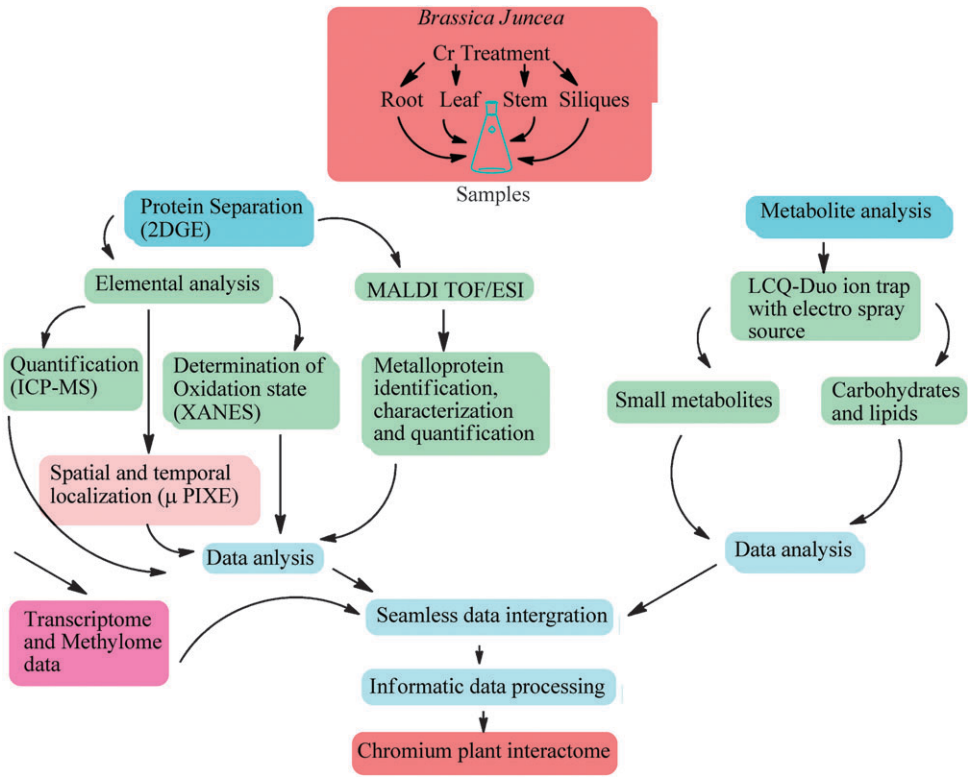

Fig. 4 Schematic representation of a proposed strategic approach for a hypothetical experiment in which proteomic and metabolomic data acquisition and integration with transcriptomic and methylome data leading to the Cr–plant interactome is highlighted.

all-inclusive quantitative and qualitative scrutiny of all the metabolites contained by a cell, tissue or organism is an extremely difficult goal and is still in its infancy in a given system, even though considerable steps forward are being made.

3.4 Bioinformatics

Reduction of the dimensionality of the data set and to envisage the data from a metallomics perspective by separating noise from signal is imperative to arrive at a wholesome picture. This would involve, apart from the algorithmic methods at every end stage of each component of the -omics study, unsupervised methods such as principal component analysis (PCA), hierarchical clustering (HCA) and K-means clustering and machine learning methods like Markov models, feature extraction and selection, and network structure deduction. Although most of the above techniques would be beyond the scope of this review a small note on a batch-learning self-organizing map (BL-SOM) would be informative. A typical example of this is given by Kim *et al.*[54] BL-SOM is an alteration of the original SOM, which provides coloured attribute self-determining maps of data input. In short, a matrix is constructed from the transcriptome and metabolome dataset in which signal intensities are ordered in various columns (experimental series) and multiple rows (gene and metabolite IDs). BL-SOM analyzes this integrated matrix of both transcriptome and metabolome data after suitable normalization of the data and initial calculations; this will give us a visual picture of the correlations between components. Genes and metabolites are classified into clusters in a two-dimensional "feature map" based on their expression and accumulation patterns.

4. Conclusions

Chromium in plants has received scant attention by researchers not only in comparison with the intense amount of interest it has generated in animal systems but also in comparison with other heavy metals in plants such as Cd, Ni, Al and As. It is time that Cr received its due notice, especially in the light of it being a therapeutic agent as well as a nutrient supplement in human nutrition, which opens up the possibility of natural supplementation against synthetic fortification[55,56] (chromium picolinate; D-phenylalanine [Cr(D-phe)]). On the other hand, chromium is still being released into the environment due to careless and inappropriate management practices of effluent discharge, mostly from industries related to metallurgy, electroplating, production of paints and pigments, tanning, and wood preservation. This has not only manifested itself in declining crop yields but also poor quality water for irrigation and human consumption. Phytoremediation, which takes advantage of a plant's natural capability to take up nutrients from the soil and the ability of the plant's cellular components to store metal ions, is an important technology to combat Cr contamination. The success of this technology involves multi-faceted interactions and so it is envisaged that sophisticated metabolic and analytical techniques should be implemented to advance the field. Genetic, protein and metabolic engineering could be the positive fallout of whole metabolome studies in Cr. However, as shown in the current status of research into

Cr plant–interactions in this brief review, whole-transcriptome profiling, proteomics and metabolomics are yet to be tapped to the fullest extent in this area. Considering the above facts there is an urgent need to reorient and refocus on research into Cr–plant interactions. The quest for a whole Cr metallome in plants will go a long way towards understanding Cr toxicity and unravelling the complete picture of interconversion of Cr species within the plant system, after their uptake, on a time course at environmentally relevant concentrations with emphasis on different stages of plant development. This would pave the way for identifying species suitable for Cr phytoremediation with qualities such as tolerance, accumulation, and biomass production.

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