Understanding saffron biology using omics- and bioinformatics tools: stepping towards a better Crocus phenome

Amjad M. Husaini1 · Syed Anam ul Haq1 · Alberto José López Jiménez2

Received: 16 September 2021 / Accepted: 6 December 2021 / Published online: 2 February 2022
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Abstract
Saffron is a unique plant in many aspects, and its cellular processes are regulated at multiple levels. The genetic makeup in the form of eight chromosome triplets (2n = 3x = 24) with a haploid genetic content (genome size) of 3.45 Gbp is decoded into different types of RNA by transcription. The RNA then translates into peptides and functional proteins, sometimes involving post-translational modifications too. The interactions of the genome, transcriptome, proteome and other regulatory molecules ultimately result in the complex set of primary and secondary metabolites of saffron metabolome. These complex interactions manifest in the form of a set of traits ‘phenome’ peculiar to saffron. The phenome responds to the environmental changes occurring in and around saffron and modify its response in respect of growth, development, disease response, stigma quality, apocarotenoid biosynthesis, and other processes. Understanding these complex relations between different yet interconnected biological activities is quite challenging in saffron where classical genetics has a very limited role owing to its sterility, and the absence of a whole-genome sequence. Omics-based technologies are immensely helpful in overcoming these limitations and developing a better understanding of saffron biology. In addition to creating a comprehensive picture of the molecular mechanisms involved in apocarotenoid synthesis, stigma biogenesis, corm activity, and flower development, omics-technologies will ultimately lead to the engineering of saffron plants with improved phenome.

Keywords Crocus sativus L. · Saffron · Genomics · Transcriptomics · Proteomics · Metabolomics · Bioinformatics

Introduction
Saffron (Crocus sativus L.) is a sterile triploid plant characterized by its red stigmas which, when desiccated, constitute the spice known as saffron. The word “saffron” is derived from “zafran”, the Arabic word that translates to “yellow”. The Crocus genus comprises over 200 recognized species of perennial geophytes. It has been proposed that C. sativus L. is an autotriploid hybrid derived from of C. cartwrightianus. C. sativus L. belongs to the Iridaceae (Liliales, Monocots) whose genomes are relatively large. From the point of view of agricultural production, Crocus sativus L. is propagated vegetatively using corms, and is prevalent throughout the tropical and subtropical regions of the northern hemisphere [1]. The major saffron growing regions of the world include Iran, Azerbaijan, Spain, Italy, India (Kashmir), Greece, and Turkey. The total world saffron production was estimated at 378.33 tons in 2016 [2], of which about 90% is produced in Iran whereas the rest of the production is located in India (Kashmir), Greece, Afghanistan, Spain and Italy [2].

Crocus sativus L. genome comprises eight chromosome triplets (2n = 3x = 24), with a genome size of 1C = 3.45 Gbp [3]. Several reports have highlighted saffron countless medicinal properties like anticancer, antimutagenic and antioxidant effects [4]. Saffron bioactive compounds have immense therapeutic properties useful for coronary artery diseases, neurodegenerative disorders, bronchitis, asthma, diabetes, fever, and colds. It has the potential to help tackle problems associated with severe acute respiratory syndrome (COVID-19) patients and post-covid-19 problems [4]. It can help manage stress and anxiety during isolation, quarantine

* Amjad M. Husaini
amjadhusaini@skuastkashmir.ac.in

1 Genome Engineering and Societal Biotechnology Lab, Division of Plant Biotechnology, Sher-e-Kashmir University of Agricultural Sciences and Technology of Kashmir, Shalimar Campus, Srinagar, Jammu and Kashmir 190025, India

2 Departamento de Ciencia y Tecnología Agroforestal y Genética, Escuela Técnica Superior de Ingenieros Agrónomos y de Montes, Universidad de Castilla-La Mancha, Albacete, Spain

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and lockdowns. Its efficacy in managing depression is comparable to drugs like imipramine, fluoxetine, and citalopram. Owing to these properties and the glamour associated with it, saffron is one of the costliest spices in the world.

Saffron is propagated through corms [5], and does not produce fertilisable gametes [6] and is self-incompatible [7]. This makes all modern saffron plants almost genetically identical. This is a bottleneck for the genetic improvement of this highly valued crop. The omics-based studies can be a benchmark for its genetic improvement [8]. These studies in saffron have broadly focused on the below-mentioned research areas.

Flower development and stigma apocarotenoid content

The most valued metabolites in *Crocus sativus* are synthesized in stigma tissue in a developmental stage-specific manner. Different stages are defined according to stigma development based on the length of the stigma, its pigmentation, and apocarotenoid content (generally yellow, orange and red in the preanthesis stigma). Almost a decade ago, we highlighted the importance of the saffron stigma transcriptome characterization for understanding the molecular basis of its flavour and colour biogenesis, the gynoecium developmental processes and flavouring compounds, and improve the quality of saffron.

Besides whole-genome sequencing, expressed sequence tags (ESTs) are a vital source for analyzing gene expression in specific organs, growth stages, developmental processes, and stress response in crops [10]. The first important database of ESTs for stigma biogenesis and apocarotenoid pathway contained 6768 ESTs [11]. The most relevant contigs included those encoding non-heme-β-carotene-hydroxylase, putative glucosyltransferase, putative isoprenoid GTases, Myb-like protein, Myb305, and Cytochrome P450 [9]. Analysis of saffron stigma EST collections at different developmental stages revealed that CsCCD2 (carotenoid cleavage dioxygenase) ESTs are predominant in the early stages [12].

Transcriptome analyses in saffron and related species (including leaves, stamens, corm, tepals, and stigmas) have uncovered a large number of transcription factor-coding genes [13–15]. The main transcripts encoding TFs upregulated in stigma are those involved in secondary metabolite biosynthesis. Among them, transcripts encoding MYB, MYB related, WRKY, C2C2-YABBY and bHLH transcription factors have been shown to be differentially expressed [15]. Besides, a total of 1075 transcripts revealed a tissue-specific expression, out of which 342 were expressed in stamen, 304 in leaf, 161 in tepal, 144 in stigma and 124 in corm.

Using deep transcriptome analysis, a novel dioxygenase carotenoid cleavage dioxygenase (CCD2), which catalyzes the first step of crocin biosynthesis from carotenoid zeaxanthin, has been identified [12]. Other studies have led to the characterization of the regulation of crocetin and crocins biosynthesis from phytoene [16]. A recent study has identified a new glycosyltransferase, UGT91P3, responsible for the last glycosylation step in the biosynthesis of crocins [17].

Genes encoding enzymes for volatile biosynthesis have been identified using in situ screening of the stigma cDNA database described previously [11, 18]. Comparison of the apocarotenoid content and expression profiles show that 1 deoxyxylulose 5 phosphatase synthase (DXS) plays a vital role in apocarotenoid accumulation. DXS is expressed at all the developmental stages of *C. sativus* stigma, while 3 hydroxy 3 methylglutaryl CoA reductase (HMGR) is expressed at low levels only. Additionally, two putative terpene synthases (TS1 and TS2) showed differential expression, with TS2 having an important role in the biosynthesis of apocarotenoids. The expression of two carotenoid biosynthesis genes, CsPSY (phytoene synthase) and CsPDS (phytoene desaturase), increases in the red stage. In another study, it was observed that during the transition from yellow to red stigmas, accumulation of zeaxanthin was accompanied by enhanced expression of phytoene synthase, phytoene desaturase and lycopene cyclase [19]. Besides, a massive accumulation of carotene hydroxylase and zeaxanthin cleavage dioxygenase transcripts was described (Fig. 1).

A systematic comparative analysis of crocin data and transcriptomes from *C. sativus*, *C. ancyrensis* and *C. cartwrightianus*, has led to the identification of putative transcription factors affecting apocarotenoid accumulation during stigma development in saffron [20]. Expression levels of DXS-CLA1, ZDS, Z-ISO, PDS, CrtISO, BCH-2, LYC-B, CCD2, and UGT74AD2 had a positive correlation with apocarotenoid levels in the three species. Besides, in stigma, eleven TFs belonging to the bHLH, C2H2, ARF, HB, CBF/ DREB1, NF-YC and ALFIN families showed correlation between expression and apocarotenoid levels in the three species. A similar study [21] compared the transcriptomes of cultivated *C. sativus* and wild *C. cartwrightianus*. This study found seven genes related to apocarotenoid biosynthesis, which showed differential expression between the samples. These genes are orthologues of carotenoid isomerase (CsTc091265), lycopene beta-cyclase (CsTc018497), zeaxanthin epoxidase (CsTc006236), UDP-glucosyltransferase (CsTc020060), phytoene synthase (CsTc009491), nine-cis-epoxy carotenoid dioxygenase (CsTc035409), and carotene beta-hydroxylase (CsTc000418). These findings provide important information for the saffron improvement
program. The orthologue of gene UDP-glucosyltransferase (CsTc020060) was down-regulated in all individual saffron plants while it is up-regulated in all the C. cartwrightianus plants [21]. UDP-glucosyltransferase, being involved in the conversion of crocetin to crocin, could be a cause behind the difference in metabolite accumulation between Crocus species. Since triploidy and sterility help safeguard the favourable allele composition (regarding aroma and colour) from being segregated by recombination, modulation of gene expression using genome modification and advanced genetic engineering approaches can be a smart strategy to increase saffron apocarotenoid content in stigma, improving saffron quality and enhancing its economic value.

Understanding saffron flower development is vital for improving its productivity and quality. The combination of class A genes (including APETALA1; CsAP1 and APETALA2; CsAP2), class B genes (including APETALA3; CsAP3 and PISTILLATA; CsPI) and class C genes (including AGAMOUS; CsAG), determines the identity of the organs developing in a whorl. An important gene in the stigma development of saffron is the C-class floral homeotic gene AGAMOUS (CsAG) [22]. Its expression began in the yellow stage of stigma, showed 16 folds increase as stigma turned from yellow to the orange stage and continued to increase up to the scarlet stage [22]. Similarly, the expression of the transcript UGT85U1 increases from yellow stage to red stage and anthesis. However, CsNCED, a regulatory gene encoding the enzyme involved in ABA biosynthesis shows lower expression in all the developmental stages [23]. Relative transcript changes of CsAP3 and NAC-like protein (CsNAP) genes have also been studied during different stages of flower development [24, 25]. However, no direct correlation in the expression of these genes could be detected. CsAP3 expression was maximum during the late pre-anthesis of stigma development, while CsNAP expression increased abruptly at the scarlet stage of stigma. The study concluded that some factor(s) could regulate CsNAP expression, while CsAP3 gene could in turn regulate the factor(s). The promoter of CsAP3 gene consists of three CArG regions, which play a pivotal role in the expression of AP3 gene, of which CArG1 is the binding site for activator proteins, thus regulating floral growth. Given this, Wafai et al. [25] conducted a study to understand the interaction between nuclear factors with B class gene CsAP3 through its CArG1 promoter region. Nuclear proteins were isolated, and a CArG1 sequence was synthesized artificially. Using Electrophoretic Mobility Shift Assay (EMSA), the binding interaction of CArG1 region with pure nuclear protein was studied, and the complex was used for protein identification using LCMS. CsNAP was identified as a conspicuous homeotic protein interacting with CArG1 region of AP3 promoter. Understanding the pathway and deciphering the complete mechanism of floral organ differentiation can pave the way for prolonged flowering of saffron by artificially manipulating the key genes. It will provide farmers ample time to collect the flowers and regulate flowering time/duration so
that flower damage caused due to early frost in November can be avoided.

In a step forward to better understand the flowering mechanism, two sets of full-length transcriptomes of flowering and non-flowering saffron crocus have been generated using NGS and SMRT sequencing [26]. Recently, morphological, physiological and transcriptome analyses of apical bud samples of C. sativus were performed during the floral transition process, and a hypothetical model for the regulatory networks of the saffron flowering transition was proposed [27].

Proteomics is central to the understanding of saffron biology. However, not much work has been reported in saffron proteomics, unfortunately. Not many data sets are available in the PRIDE PRoteomics IDEntifications (PRIDE) Archive database [28]. The first dedicated protein database for saffron stigma (Crocus sativus L, taxonomy-id: 82528) samples at different developmental stages have been created only in the recent past [29]. The MS proteomics data can be accessed from the ProteomeXchange Consortium via the PRIDE partner repository with the data set identifier PXD009014 (https://www.ebi.ac.uk/pride/archive/projects/PXD009014). In another recent study, protein profiling of flowering and non-flowering saffron buds subjected to cold stress was done using isobaric tags for relative or absolute quantitation (iTRAQ). Out of 5624 proteins identified in the study, 201 were differentially abundant protein species (DAPs) between these two groups. Upregulated DAPs play an important role in sucrose metabolism, lipid transport, glutathione metabolism, and gene silencing by RNA. Downregulated DAPs are involved in starch biosynthesis and oxidative stress response. Three new flower-related proteins, CsFLK, CseIF4a, and CsHUA1 were identified too [30].

A search in the GenBank protein database for saffron leads to just over 530 entries, these entries correspond to C. sativus (268), C. cartwrightianus (258), and C. ancyrensis (4) (http://www.ncbi.nlm.nih.gov/). Despite several tools available for predicting and visualising secondary and tertiary structures of proteins, there is no detailed analysis in saffron. A search on saffron crocus query in the UniProt Knowledgebase (UniProtKB) returns only 426 entries, out of which 420 are in Unreviewed (TrEMBL). Only six have been manually reviewed in Swiss-Prot, and include Crocetin glucosyltransferase 2, Crocetin glucosyltransferase 3, Profilin, Zeaxanthin 7,8(7′,8′)-cleavage dioxygenase, Carotenoid 9,10(9′,10′)-cleavage dioxygenase, and Pollen allergen Cro s 1. We could find only one 3-D X-ray diffraction-based crystal structure of saffron protein in the protein data bank (PDB) viz. Cysteine Protease (at 1.3 A Resolution) and is available at http://www.rcsb.org/pdb/explore/explore.do?structureId=3U8E.

As already highlighted, unlike rice, maize, wheat, or tomato, there are limited saffron-specific genomic resources available to explore its peculiar biology. There is a need to explore and utilize most modern technologies that can generate maximum useful information. Activity-based protein profiling (ABPP) is one such novel techniques of chemical proteomics that has recently revolutionized proteomics. Besides its use in drug selectivity and diagnostics, its application in plant science has grown in the last few years [31]. ABPP uses small molecules as probes for labelling enzymes when these are in an active state. In saffron, the first report on ABPP demonstrated the multiplexing of probes and generated useful information about the active proteases involved in the different developmental stages of stigma [29]. This approach has successfully identified and quantified sixty-seven differentially active glycosidases during the stigma development, implying that glycosidase activity is vital for stigma maturation. These results suggested potential candidate glycosidases involved in the conversion of picrocrocin into safranal.

GOLM and the MASSBANK are popular databases for metabolomic profiling, providing information of reference mass spectra from biologically active metabolites. Other databases like Kyoto Encyclopedia of Genes and Genomes (KEGG), Reactome, MetaCyc and GO-ontology are important resources for curated information regarding biochemical pathways and molecular functions wherein these metabolites perform specific roles. Studying the flavonoid glucosylation and carotenoid biosynthesis enzyme metabolomics [18, 32] is vital for understanding the dynamics of these pathways in saffron. Metabolic analysis of stigma at yellow stage has shown low levels of crocetin, crocins, picrocrocin, and some unidentified compounds with maximum wavelengths around 250 nm. Picrocrocin and crocins have been detected early in the orange stage, increasing rapidly in the red stage. The glycosylated products of crocetin reach maximum levels in the red stage [33]. Picrocrocin level rises in the orange stage and achieves the maximum level at anthesis [34].

Besides apocarotenoids, saffron contains volatile compounds also. More than 160-volatile compounds have been detected using chromatography, spectroscopy and mass spectrometry techniques [35, 36]. In the yellow stigma (stage), the fatty acid derivatives predominate, while in the orange (stage), carotenoid derivatives too are present in addition to the fatty acid derivatives. In the red stigma (stage), the volatiles derived from carotenoids accumulate to high levels, and β-cyclocitril, generated by the cleavage of β-carotene reaches maximum level. Just before anthesis at the scarlet stigma (stage), the volatile propanoic acid, 2-methyl-2,2-dimethyl-1-(2-hydroxy-1-methylethyl) propyl ester accumulate at high levels. However, their levels decrease at anthesis when monoterpenes and carotenoids reach their maximum levels [10]. Among the monoterpenes, linalool is emitted at high levels at anthesis and is responsible for floral odours [37]. In the post-anthesis stage, the fatty acid-derived volatiles become the main volatile compounds.
Diversity of saffron and its characterization

Despite the advancement of sequencing technology and its affordability, there is no whole-genome sequence available for any Crocus species. Some classical cytogenetic analyses involving chromosome counting and karyotyping have been done in saffron [38]. Those studies have shown that saffron is a triploid with karyotype $2n = 3x = 24$. It comprises of 8 triplets: two triplets are sub-acrocentric, three triplets are metacentric, two triplets are sub-metacentric and one triplet contains two kinds of chromosomes: chromosome 5(1), metacentric, and chromosomes 5(2,3), sub-acrocentric and smaller [10]. Some efforts have been made to improve our understanding of the genomic organisation of Crocus species. These studies are mostly based on Random amplified polymorphic DNA (RAPD) [39], Inter-Retrotransposon Amplified Polymorphism (IRAP) markers [40, 41], Amplified Fragment Length Polymorphism (AFLP) and Simple Sequence Repeats (SSR) [42].

The barcode analysis of the 86 species of genus Crocus using rpoC1, matK and tmH-psbA regions has shown the importance of barcodeing in the genetic diversity of Crocus [43]. RAPD and inter simple sequence repeat (ISSR) marker profiles of 43 isolates of C. sativus collected from different geographical areas have been used to determine if this species is monomorphic or polymorphic. The results showed that the clones were identical at the molecular level [44]. Surprisingly, ISSR markers showed no differences between C. sativus and C. cartwrightianus [45]. In contrast, RAPD markers revealed considerable genetic diversity among 10 elite saffron clones selected in Kashmir [46]. Long terminal repeats (LTRs), a retrotransposon (RTN)-based marker study in Iranian species of Crocus showed high diversity within and between species [40]. Using 12 microsatellite markers [47] succeeded in detecting good polymorphism within fifty Iranian individuals of Crocus sativus. A reasonable amount of polymorphism was detected in similar studies among Iranian C. sativus germplasms [48].

There is ample evidence that epigenetics plays an important role in creating inheritable variation contributing significantly to different traits in several plant species. DNA methylation is the most widely studied epigenetic mark in plants as its genome-wide investigation is easier to accomplish [49]. In a study involving more than a hundred saffron accessions from WSCC (World Saffron and Crocus Collection, Spain), very low genetic variability was detected using 12 AFLP (Sensitive Amplified Fragment Length Polymorphism) primer combinations. In contrast, very high epigenetic variability was detected with just 3 Methylation-Sensitive Amplified Fragment Length Polymorphism (MS-AFLP) primer combinations [50]. Five accessions from the WSCC germplasm having extremely low genetic variability were cultivated for 3 years in the same field. These accessions of different origins maintained different epigenotypes. This suggests that the epigenetic structure of saffron is highly stable [51]. The stability of saffron epigenotype over the years supports the idea that epigenetics may play a vital role in the constancy of saffron phenotype variability.

AFLP analysis using methylation-sensitive restriction enzyme-sequencing (MRE-seq) gave more insight into saffron’s epigenome [52]. The study compared the epigenetic profile of 5 phenotypically different, but genetically similar accessions from WSCC germplasm. Differential methylation of regions was detected in some genes encoding transcription factors, shaping the alternative phenotypes. Many SNPs and INDELs were identified, showing thereby that genetic polymorphism exists within the saffron species. Genetic variants were also detected in Gene Ontology (GO) terms, portraying a genetic basis for alternative phenotypes. A heatmap of the 50 highest polymorphic GOs shared between accessions highlighted the presence of two distinct clusters of Indian and Spanish accessions. Twelve GOs showed lower polymorphism in the Spanish accessions than Indian accessions [52].

Phylogenetic analyses of nuclear loci and chloroplast genome, as well as genome-wide DNA polymorphism, indicate that C. sativus is genetically closer to C. cartwrightianus populations. Genome sequencing and Fluorescence in situ hybridisation (FISH) have demonstrated that genomes of two C. cartwrightianus individuals with slight chromosomal differences had gotten fused, and it could be the parental origin of saffron C. sativus L. [53]. Another view is that the most likely ancestors of saffron are C. cartwrightianus and C. pallasii subsp. Pallasii (or close relatives) [54].

Saffron growth, development and disease

While there are ample omics-based studies on apocarotenoid biosynthesis pathway, the studies on saffron growth and development are limited. Proteomic analysis has led to the identification of differentially accumulated proteins (in somatic embryos) of C. sativus. Thirty-six proteins have been identified, including those involved in protein synthesis, carbohydrate and energy metabolism, defence and stress response, nitrogen metabolism, and secondary metabolism [55]. Metabolomic studies have provided insights into the corm composition of C. sativus, too [56]. At the sprouting stage (in corms), sugars like glucose, fructose, and maltose reveal a strong positive correlation with palmitate, turanose, oxalic acid, ethanolamine, linoleic acid, and tetranoic acid; and a negative correlation with sitosterol mannoside and
octadecanoic acid. At bud development, fatty-acid biosynthesis significantly relied on carbohydrate metabolism intermediates. Sucrose breakdown reached its maximum to begin the sprouting and bud growth in *C. sativus*.

Climate change and the associated biotic and abiotic stresses are the most daunting challenges to saffron cultivation [8]. Omics-based biological studies of saffron crop shall pave the way for its sustainable production, especially given the problems associated with climate change. MicroRNAs of plants, though ubiquitous and small in size, play an important role in abiotic stress. MicroRNA sequencing, yet ignored in *C. sativus*, can be vital in understanding the regulation of saffron genomic elements. These can also throw light on the regulatory networks underlying the apocarotenoid biosynthesis in *C. sativus*. A study on an EST library from mature *C. sativus* stigmas has helped detect two putative microRNAs, miR414 and miR837-5p, in saffron stigma [57]. Co-expression network analysis has revealed them playing vital roles in metabolic pathways. The predicted targets of the miR414 are: β-carbonic anhydrase 5, Transducin/WD40 repeat-like superfamily protein and three-transposable element genes AT2G13700.1, AT4G06613.1, AT3G29783.1. The predicted targets of miR837-5p are SEC14 cytosolic factor family protein/phosphoglyceride transfer family protein, enhancer of polycomb-like protein, and F-box/RNI-like/FBD-like domains containing protein. In addition, three more miRNAs, csa-miR1, csa-miR2 and csamiR3 have also been predicted by using in silico methods of EST analysis [58]. The predicted targets of these miRNAs are involved in regulating plant growth, senescence, stress responses, disease resistance, mRNA export, protein synthesis and post-translational modifications [58].

In an RNA-seq based transcriptome study, useful information was categorised in the form of small databases for viruses, bacteria, fungi, and plants [59]. It used YeATS suite from the NCBI and Ensembl databases, and showed that the soybean mosaic virus is abundantly expressed in the corm, tepal, leaf, stigma, and stamen tissues [59]. Furthermore, it has been shown that there is a difference in fungal diversity between roots and corms of *C. sativus*. At the flowering stage, the dominant phylum in the rhizosphere is Zygomyctota, while in the cormosphere Basidiomycota is dominant. In the cormosphere, Basidiomycota is prevalent at the flowering stage, while Zygomyctota is dominant at the dormant stage. However, in the bulk soil, Ascomycota dominates during both stages [60].

Saffron corm rot caused by *Fusarium oxysporum* is a major disease, causing heavy losses in saffron-producing countries [8]. ABPP, a chemical proteomics-based technique, has been upscaled by multiplexing diverse probes (targeting serine hydrolases, α-glycosidases, β-glycosidases and cysteine proteases) to give a broad snapshot of active proteases having a role in corm rot infection [29]. The suppressed activity of an α-glycosidase upon *F. oxysporum* infection has been detected, which is consistent with the view that *F. oxysporum* suppresses AGLU1 in the apoplast to overcome its antifungal activity [29]. While the activities of putative α-glycosidases (100-kD) and β-glucosidases (50–70 kD) increased upon infection, the activities of serine hydrolases (50, 60 kD) decreased. Additionally, many β-glucosidases (45–60 kD) appeared, while some (65–70 kD) disappeared. In the ABPP based chemical proteomics study, drastic changes were visualised in the activity profile of cysteine proteases, especially papain-like Cys proteases and vacuolar processing enzymes (Table 1).

### Saffron adulteration and spice quality

The molecular analysis involving a complete set of metabolites existing in a cell at a particular instant is the backbone of understanding metabolic pathways and is called metabolomics. It is highly significant for plants due to the crucial role of the secondary metabolites in plant survival. These metabolites are extracted from the tissues, separated and analysed in a high-throughput manner to generate metabolic fingerprints. Many tools available in the bioinformatics toolbox help identify and characterise these metabolites. In saffron, metabolite fingerprinting (based on 1H NMR spectra) and chemometrics have helped in authenticating saffron as Italian or Iranian [61]. These techniques also helped to detect the presence of plant-based adulterants in saffron [62]. 1H NMR and chemometrics studies have shown that saffron can preserve its valuable characteristics up to 4 years [63].

High-performance thin-layer chromatography (HPTLC) helps study chemical diversity among saffron accessions. In a recent study, 53 saffron accessions from Khorasan Razavi were characterized for chemical diversity using HPTLC. Based on the heat maps generated at different wavelengths, crocin and picrocrocin content was found helpful in categorising saffron [64]. The third important bioactive molecule, safranal, is not among the major volatiles produced in the fresh tissue. Safranal, which gets produced by picrocrocin degradation during the dehydration of the stigma, is the primary aroma component comprising 60–70% essential oil content [65].

### Medicinal value and drug development

Saffron bioactive compounds have immense therapeutic properties, including those beneficial against coronary artery diseases, neurodegenerative disorders, bronchitis, asthma, diabetes, fever, colds, and metabolic syndrome. A detailed analysis of its medicinal properties points to its immense untapped potential for easing the distress symptoms of
### Table 1  Significant research findings and outcomes of omics-based research studies conducted in saffron and its allies

| S. no. | Omics approach used | The gist of the main findings and outcomes | References |
|--------|---------------------|--------------------------------------------|------------|
| 1.     | Genomics            | Whole genome sequencing of *Crocus* sp. has not been done<br>There are contradictory results on the detection of polymorphisms using marker-based analysis<br>Some studies conclude that saffron is a monomorphic species and whole genome sequencing is needed to discriminate between its isolates<br>Some studies show that molecular markers are quite efficient in detecting polymorphism. Such studies conclude that saffron is not monomorphic and that there is diversity which could be useful for breeding purposes<br>AFLP analysis using methylation-sensitive restriction enzyme-sequencing (MRE-seq) has shown that phenotypically different but genetically similar accessions vary in the methylation pattern of genomic regions encoding transcription factors and may result in alternative phenotypes<br>Epigenetic structure in saffron is highly stable and may play a vital role in the constancy of saffron phenotype variability<br>ISSR primers are reported to be capable of easily distinguishing genuine saffron from fake one | [44, 47, 48, 51, 52, 54, 88–91] |
| 2.     | Transcriptomics     | De novo transcriptome assemblies have been created from leaves, stamens, corm, tepals, and stigmas of *Crocus sativus*<br>The most valued compounds of *C. sativus* are synthesised inside stigma in a developmental stage-specific manner<br>During the transition from yellow stage to red stage stigmas there is an accumulation of zeaxanthin accompanied by sharp increase in the expression of phytoene synthase, phytoene desaturase, lycopene β cyclase, β carotene hydroxylase and zeaxanthin cleavage dioxygenase<br(CsCCD2) (carotenoid cleavage dioxygenase) ESTs are prominent in the saffron stigma libraries obtained from early stages of stigma development<br>UDP-glucosyltransferase is vital for conversion of crocetin to crocin, and therefore causes difference in metabolite accumulation between Crocus species<br>1 Deoxyxylulose 5 phosphate synthase (DXS) plays a vital role in apocarotenoid accumulation in stigma<br>There is no direct concordance in the expression of CsAP3 and CsNAP gene expression in saffron<br>Identification, isolation, and biochemical characterisation of uridine diphosphate glycosyltransferase (UGT709G1), which catalyses the HTCC glucosyltransferase reaction to yield picrocrocin, can provide a vital lead for the industrial production of picrocrocin/safranal<br>Differentially expressed full-length transcripts of flowering and non-flowering saffron crocus have been identified and characterised<br>Stigma development in field- and indoor-cultivated saffron is similar with respect to apocarotenoid content and gene expression profiles of 12 genes involved in apocarotenoid biosynthesis<br>Carotenoid cleavage dioxygenase (CCD2) catalyzes the first step of crocin biosynthesis from carotenoid zeaxanthin and gets expressed at an extremely high level in the stigma as compared to corm, leaf, tepal, and stamen<br>A C-class floral homeotic gene AGAMOUS (CsAG) gene is vital for stigma development of saffron. Its expression begins at yellow stage of stigma and increases sharply to orange stage, and continues to increase upto scarlet stage<br>CsAP3 expression is maximum at late preanthesis of stigma development, while CsNAP expression increases abruptly at the scarlet stage of stigma<br>CsNAP protein binds to the CArG1 region of CsAP3 promoter, and might be regulating CsAP3 expression indirectly by modulating CArG1 promoter | [9–12, 14, 15, 18–27, 72, 92–95] |
Table 1 (continued)

| S. no. | Omics approach used | The gist of the main findings and outcomes | References |
|--------|---------------------|------------------------------------------|------------|
| 3.     | Metabolomics        | Two novel saponins namely Azafrine 1 and Azafrine 2 have been isolated, purified, and structurally elucidated from the external part of saffron corm, suggesting that they may be acting as phytoprotectans. | [4, 33, 56, 63, 64, 96–101] |
|        |                     | $^1$H NMR-based metabolomics is useful to determine quality deterioration of saffron upon storage and for quality control. | |
|        |                     | Liquid chromatography coupled to electrospray ionisation time-of-flight mass spectrometry is an important tool for assessing saffron authenticity. | |
|        |                     | Tepals may have nutrition value owing to the presence of phytosterols and fatty acids, and can be processed as a source of flavonoids. | |
|        |                     | Metabolite profiling of stigma, tepal and stamen of *Crocus sativus* flower by ultra-performance liquid chromatography-quadrupole time-of-flight mass spectrometry (UPLC-QToF-MS/MS) has shown that coniferin and crocin-2 are special components in stigmas, while flavonoids are high in tepals. | |
|        |                     | High resolution mass spectrometry metabolomic studies in saffron from several countries has revealed that the phytochemical content varies among the samples of different countries. | |
|        |                     | At the yellow stage of stigma there are very low levels of crocetin, crocins, picrocrocin. | |
|        |                     | Picrocrocin and crocins are detected early in the orange stigma stage and increase rapidly in the red stigma stage. | |
|        |                     | The glycosylated products of crocetin reach maximum levels in the red stigma stage. | |
|        |                     | Saffron bioactive compounds are useful against coronary artery diseases, neurodegenerative disorders, bronchitis, asthma, diabetes, fever, colds, and metabolic syndrome. | |
|        |                     | Saffron can alleviate the symptoms of severe acute respiratory syndrome coronavirus 2 (COVID-19) patients and manage post-covid-19 syndrome. | |
|        |                     | The efficacy of saffron in managing depression is comparable to drugs like imipramine, fluoxetine, and citalopram. | |
|        |                     | Saffron can be used as an adjuvant in drug formulations as it acts as an immuno-booster and anti-depressant. | |
| 4.     | Proteomics          | Thirty-six differentially accumulated proteins have been detected during somatic embryogenesis in *Crocus sativus* and involvement of ascorbate–glutathione cycle has been suspected in somatic embryo establishment. | [29, 30, 55] |
|        |                     | Saffron protein database of stigma at different developmental stages is available through ProteomeXchange Consortium via the PRIDE partner repository with the data set identifier PXD009014 https://www.ebi.ac.uk/pride/archive/projects/PXD009014 | |
|        |                     | Two hundred and one differentially abundant protein species (DAPs) under cold stress affecting the floral initiation of saffron have been revealed using iTRAQ-based proteomics followed by real-time qPCR. | |
|        |                     | Saffron dormant corms exposed to low temperature stress do not bloom perhaps due to changes in the ‘reactive oxygen species–antioxidant system–starch/sugar interconversion homeostasis flowering pathway’ | |
| 5.     | ABPP                | Drastic changes in the activity profile of cysteine proteases especially papain-like Cys proteases and vacuolar processing enzymes occur in the corms infected with *Fusarium oxysporum*. | [29] |
|        |                     | The activity of α-glycosidase AGLU1 gets suppressed upon *Fusarium oxysporum* infection in saffron corms irrespective of the F.o strain. | |
|        |                     | Activities of putative α-glycosidases (100-kD) and β-glycosidases (50–70 kD) increase upon *F. oxysporum* infection, while the activities of serine hydrolases (50, 60 kD) decrease. | |
|        |                     | Many β-glycosidases (45–60 kD) appear, while some (65–70 kD) disappear during *F. oxysporum* infection. | |
|        |                     | Glycosidase activity has a major role in maturation and development of stigma. | |
|        |                     | Sixty-seven active glycosidases that are differentially active during stigma development have been identified and quantified. | |
severe acute respiratory syndrome coronavirus 2 (COVID-19) patients and managing the post-covid-19 syndrome [4]. Despite the importance of saffron in medicine and phytochemistry, modern approaches based on omics studies are relatively rare [3, 41, 42].

The metabolic and biochemical properties of saffron confirm its immense role in the pharmacognosy and pharmaceutical industry [4]. Studies on the binding potential of carotenoid pathway bioactive molecules for angiotensin-converting enzyme 2 (ACE2) receptor of SARS-CoV-2 show the possibility of using the saffron-based remedy for novel coronavirus [66]. Flexible molecular docking followed by atomic level interaction study indicated that lutein and picrocrocin form various interactions with different amino acid residues of ACE2. In-depth analysis revealed that these interactions could be crucial for receptor-binding domain (RBD) binding and, therefore, potentially disrupting the interaction between RBD and ACE2. The study provides a clue for advanced studies involving in vitro, animal models and clinical studies. The efficacy of saffron in managing depression is comparable to drugs like imipramine, fluoxetine, and citalopram. The saffron metabolites can help manage stress and anxiety during the prolonged lockdown, isolation, and quarantine. Owing to all these beneficial properties and as an immunity booster, saffron extracts may be added in some drug formulations in future.

Bioinformatics for omics data analysis

Intricate regulatory networks of gene expression control the tissue and stage-specific accumulation of various metabolites. The systems biology approach integrates different omics technologies, including transcriptomics, proteomics, and metabolomics, so that biological systems are investigated in an integrated manner at different levels. The analysis of the complexity of the generated datasets needs to be integrated into the framework of known biological pathways. Bioinformatics plays a crucial role in data generation, analysis, and interpretation of the different omics technologies when mining meaningful information. It is crucial to interpret the massive amount of data generated through high throughput technologies and filter out useful information for interpretation by the researchers for comprehensive views on systems functionality [67]. Moreover, it provides resources derived by exploiting -omics technologies or subsequent analyses, including sequence comparisons, gene family investigations, and molecular modelling, among others [68].

Omics-based technologies and other molecular research tools have led to the generation of a huge amount of information, necessitating bioinformatics advancement. It acts like a ‘feedback promotion’ and causes advancement in omics technologies due to its better handling of the ‘big data’. Bioinformatics creates and advances algorithms, computational techniques, and databases to better solve problems in the analysis of huge biological data. It has a key role in the textual mining of biological literature and query biological information. Bioinformatics tools can easily compare genetic and genomic data to better understand the evolutionary relationships between organisms. At a more integrative level, it analyzes the biological pathways and metabolic networks to give a better understanding into the systems biology. It helps in conducting simulation and modelling studies on DNA, RNA and proteins to understand their molecular interactions better, thus strengthening structural biology. It has assisted evolutionary biologists to (i) trace the evolution of organisms by calculating changes in their DNA; (ii) build complex models of populations for predicting the outcome; and (iii) share information about a large number of species [69]. The most relevant bioinformatics tools that have contributed to recent studies on saffron biology have been summarized in Table 2.

Large-scale expression profiling studies in saffron have generated huge amounts of data, and the discipline of bioinformatics has been indispensable for ‘deriving information’ from these data. As predicted [9], characterisation of the saffron stigmas through omics studies coupled with bioinformatics tools has generated vital novel information about the molecular basis of flavour, colour biogenesis, genomic organization, and the biology of Crocus gynoeccium (Table 1).

GenoType and GenoDive are two important programs for analyzing genotypic diversity in clonal/asexual organisms [70]. The significance of genetic differentiation between accessions of saffron in Iran through the calculation of clonal diversity indices and Analysis of molecular variance (AMOVA) has been done using these tools [50]. Plant Intron and Exon Comparison (PIECE) is a comprehensive plant gene comparison and evolution database containing all the annotated genes described from 25 plant species with
| S. no. | Bioinformatic tools          | Web address                                           | Role                                                                 | References |
|-------|-----------------------------|-------------------------------------------------------|----------------------------------------------------------------------|------------|
| 1     | SAM and BCF tools           | https://www.htslib.org/                               | Tools for processing and analysing sequencing data                    | [102]      |
|       |                             | https://github.com/samtools/samtools                  |                                                                      |            |
|       |                             | https://www.htslib.org/                               |                                                                      |            |
|       |                             | https://github.com/samtools/bcf tools                  |                                                                      |            |
| 2     | MEGA                        | http://www.megasoftware.net/                          | Comparative analysis and inferring evolutionary relationships of homologous sequences | [103–106] |
| 3     | Trinity                     | https://github.com/trinityrnaseq/trinityrnaseq/releases/tag/v2.8.6 | Tool for de novo transcriptome assembly of RNA-seq data               | [87, 107] |
| 4     | SMART 9                     | https://smart.embl.de/                                | Database for Identification and analysis of protein domains within protein sequences | [108]      |
| 5     | MPI bioinformatics toolkit  | http://toolkit.tuebingen.mpg.de/                      | Web service for comprehensive and collaborative protein bioinformatic analysis | [109, 110] |
| 6     | BiGGEsTS                    | http://kdbio.inesc-id.pt/software/biggests             | Tool for revealing local coexpression of genes in specific intervals of time | [111]      |
| 7     | PlantGDB                    | http://www.plantgdb.org/                              | Database for comparative genomics/ genomic database encompassing sequence data for plants | [112]      |
| 8     | KEGG                        | http://www.kegg.jp/                                  | Database resource for biological interpretation of genome sequences and other high-throughput data | [80]       |
|       |                             | http://www.genome.jp/kegg/                           |                                                                      |            |
| 9     | TrichOME                    | http://www.planttrichome.org/                         | Comparative Omics database for plant trichomes                        | [113]      |
| 10    | PlantTFcat                  | https://www.zhaolab.org/PlantTFcat/                   | Tool for Identification and categorisation of plant transcription factors and transcriptional regulators | [114]      |
| 11    | Pln TFDB                    | http://plntfdb.bio.uni-potsdam.de/v3.0/               | Database for functional and evolutionary study of plant transcription factors | [115, 116] |
| 12    | Ensembl Plants              | http://plants.ensembl.org                             | Database for visualising, mining and analysing plant genomic data     | [117]      |
| 13    | Wego                        | http://wego.genomics.org.cn/                          | Web tool for plotting GO annotations                                  | [118]      |
| 14    | edgeR                       | http://bioconductor.org/packages/edgeR/               | Package for differential expression analysis of digital gene expression data | [119, 120] |
| 15    | Bowtie                      | http://bowtie.ccbu.umd.edu/                           | Ultrafast, memory-efficient alignment program for aligning short DNA sequence reads to large genomes | [121]      |
|       |                             | https://sourceforge.net/projects/bowtie-bio/          |                                                                      |            |
| 16    | KaPPA-View                  | http://kpv.kazusa.or.jp/kpv4/                          | Web-based database for analysing omics data in plants                 | [122, 123] |
| 17    | Transcriptogramer           | http://bioconductor.org/packages/transcriptogramer    | R package for transcriptional analysis based on protein–protein interaction | [124]      |
| 18    | Cufflinks                   | http://cole-trapnell-lab.github.io/cufflinks          | Open-source software for RNA-Seq data analysis                        | [125, 126] |
| 19    | Paintomics                  | http://www.paintomics.org/                            | Web based tool for joint visualization of transcriptomics and metabolomics data | [127]      |
| 20    | PIECE                       | https://probes.pw.usda.gov/piece/index.php            | Database for plant gene structure comparison and evolution            | [128]      |
| 21    | MISA-Web                    | https://misaweb.ipk-gatersleben.de/                   | Tool/web server for microsatellite prediction and counting            | [129]      |
| 22    | Prodigal                    | https://github.com/hyattpd/Prodigal                   | Protein-coding gene prediction software tool                          | [83]       |
| 23    | GeneMarkS-T                 | http://topaz.gatech.edu/GeneMark/license_download.cgi | Tool for identification of protein-coding regions in RNA transcripts  | [84]       |
| 24    | MaxQuant                    | https://maxquant.net/maxquant/                        | Quantitative proteomics software package for analysing large mass-spectrometric data sets | [86]       |
| 25    | Perseus                     | https://maxquant.net/perseus/                          | Software platform for interpreting protein quantification, interaction and post-translational modification data | [130]      |
available sequenced genomes, allowing a comparative analysis of saffron gene structures [71]. MicroSatellite (MISA) microsatellite finder is a tool for finding microsatellites in nucleotide sequences. Using this Perl script in saffron [72] has led to the identification of microsatellites, also known as simple sequence repeats (SSRs), in *C. sativus*.

As *C. sativus* is a species without whole-genome sequencing, de novo transcriptome analysis provides an excellent and necessary platform to deepen the research on this plant at the molecular level [73]. Full-length reconstruction of transcriptomes from short-reads generated by Illumina sequencing technologies is the most challenging step in RNA-seq studies. In the absence of a reference genome, most common assembly strategies rely on Bruijn graph, including packages such as Trinity, SOAPdenovo-Trans, Velvet, Rnnotator and Oases [74]. Many studies have used Trinity for de novo assembly of saffron transcriptomics data [27, 73, 75], whereas others rely on strategies combining some of the aforementioned packages [15]. Alternative methods to Illumina sequencing, such as PacBio long-read sequencing, imply specialized software such SMRT Analysis software suite [26, 72, 76].

One of the main downstream applications after de novo assembly is transcript expression estimation which, in the absence of a reference genome, is performed by mapping reads against the assembled transcriptome. Algorithms that quantify expression from transcriptome mappings include RSEM, eXpress, Sailfish and kallisto, among others [74]. These algorithms typically depend on short read alignment programs such as Bowtie, which enables ultrafast and memory-efficient alignment of large sets of sequencing reads to a reference sequence [77]. In a recent study, the differentially expressed genes in saffron were identified via pair-wise comparisons of gene expression patterns between stigma and the other four tissues (corm, leaf, tepal and stamen) by the ‘DESeq’ package [72]. The package is used for quantitative analysis of comparative RNA-seq data using shrinkage estimators for dispersion and fold change.

Functional annotation of the transcripts generated by the above-mentioned methods is typically achieved using similarity-detection tools such as BLAST [78]. Blast2GO has become a popular tool, allowing massive annotation of complete transcriptome datasets against a variety of databases, as well as GO functional classification and KEGG pathway enrichment [79]. Other software, WEGO (Web Gene Ontology Annotation Plot), allows visualizing, comparing and plotting GO annotation results. These tools have been widely used in the functional classification of unigenes in RNA-seq studies on *Crocos sativus* [13, 20, 27, 32, 72]. Other annotation tools are based on the identification of specific domains in protein sequences. PlantTFcat is a high-performance web-based analysis tool that is designed to identify and categorise plant Transcription factor (TF)/Transcriptional regulator (TR)/Chromatin regulator (CR) genes from genome-scale protein and nucleic acid sequences by systematically analysing InterProScan domain patterns in protein sequences. Candidate transcription factors implicated in crocin biosynthesis in *Crocos sieberi* tepal and *C. sativus* stigma have been identified using PlantTFcat [13]. The Plant Transcription Factor Database (Pln TFDB), which is an integrative database that provides putatively complete sets of transcription factors and transcriptional regulators in plant species, has been used to identify genes encoding transcription factors in the network in saffron [57].

RNA-sequencing is a valuable tool to gain knowledge on high-level functions in biological systems. KEGG is an integrated database resource for biological interpretation of genome sequences and other high-throughput data [80]. It is
the reference knowledge base that integrates current knowledge on molecular interaction networks such as pathways and complexes (PATHWAY database), information about genes and proteins generated by genome projects (GENES/SSDB/KO databases) and information about biochemical compounds and reactions (COMPOUND/GLYCAN/REACTION databases) [81]. It has been used in Crocus sativus L. protein domain annotation [29, 72]. Open reading frame detection and domain annotation from de novo assembled transcripts of Crocus sativus L. using TransDecoder along with two other algorithms (GeneMarkS-T, Prodigal) has led to the identification of 67 active glycosidases that are differentially active during stigma development, implying that glycosidase activity has a major role in the maturation of stigma [29]. Prodigal (PROkaryotic DYnamic programming Gene-finding Algorithm) is a fast, lightweight, open-source gene prediction program [83], while GeneMarkS-T is used for ab initio identification of protein-coding regions in RNA transcripts [84].

Different proteins that were either upregulated or downregulated in saffron under cadmium toxicity have been putatively identified using the MASCOT software search engine [85]. MaxQuant is a proteomics software for analysing large mass-spectrometric data sets [86]. It has been used for peptide and protein identification in different developmental stages of saffron stigma [29]. Peptide relative quantification between different MS runs was based solely on the LFQs, as calculated by MaxQuant (MaxLFQ algorithm). Another associated software platform (Perseus) supports researchers in the interpretation of protein quantification, interaction and post-translational modification, and is used for statistical analysis of MaxQuant outputs [86]. Saffron stigma spectra files submitted to an Andromeda search in MaxQuant were finally analysed and filtering of the results was done for post-translational modification, pattern recognition, time-series analysis in Perseus version 1.5.5.3. [29].

As discussed above, the identification and quantification of active glycosidases using ABPP could not have been possible without the support of bioinformatics tools [29]. Open Reading Frame Detection and Domain Annotation Softwares like Gene-MarkS-T [84], TransDecoder [87] and Prodigal [83] were used. Eight glycosidases (three GH3, three GH35, two GH116, and one GH1) were up-regulated more than twofold in stage 4 stigmas. Moreover, the differential 110-kD βGH (Glucoside hydrolase) detected with labeling is most likely the GH116 enzyme CsTc017194, because this enzyme has a predicted molecular mass of 106 kD and is 4.5-fold up-regulated in stage 4 stigmas. The study illustrates the power of ABPP with bioinformatic predictive algorithms for quantitative glycosidase activity profiling on non-model plant species, like Crocus sativus L.

As shown by all these studies, there is immense scope for bioinformatics on elucidating biochemical functions of saffron proteins and its bioactive compounds [4, 29, 66].

Future prospects of saffron genetics

The studies cited above are a clear example of the tremendous advances in understanding the biology of saffron in recent years, yet there are many challenges and opportunities ahead. Deeper understanding of biological pathways has taken us closer to achieving the goal of developing a genome engineered Crocus sp. that can yield better quality saffron with high productivity. It will not be too far when these techniques enable editing genes encoding apocarotenoid biosynthesis through novel genome editing tools like CRISPR-Cas, making saffron breeding programs successful. In this context, whole-genome sequencing of C. sativus L. is becoming increasingly necessary to facilitate the exploitation of available gene-editing technologies.

Omics tools can be useful in locating sources of resistance and agronomically interesting traits for transfer to saffron by appropriate biotechnological tools [8] so that the resulting phenotype is able to better survive the challenges of erratic weather patterns and stresses of climate change. Such tools can also help appreciate the extent of the diversity of various geographic or genetic groups of cultivated saffron to infer relationships between groups and accessions. The information derived can be utilised for constructing biological pathways involved in the biosynthesis of principal components of saffron. Saffron metabolomics studies have revealed many peculiar properties of this interesting spice. However, the major challenge remains in identifying the incongruities in the biochemical pathways and the metabolic networks and correlating them with the phenotype.

Conclusion

Omics-based technologies have revolutionized biology, and saffron is no exception. These studies have helped to better understand the molecular mechanisms of flower development and growth, as well as the agents involved in apocarotenoid biosynthesis, its quality and diversity, pathology, and its potential as a therapeutic agent. Except for saffron
whole-genome sequencing, which is still awaited, a lot of useful information about saffron biology has been generated using omics-based techniques. These novel technologies helped discover new genes, study their expression, function, and evolutionary relationships, and made a plethora of information available to the scientific community, eventually taking us closer to developing a better quality high yielding saffron Crocus sp.

Acknowledgements AMH is grateful to National Mission on Himalayan Studies, Ministry of Environment, Forest and Climate Change, Government of India for funding saffron research in the form of a research grant to him. He is also thankful to the Scientists and staff of SKUAST-K for their help and support.

Funding Not applicable.

Data availability Not applicable.

Code availability Not applicable.

Declarations

Conflict of interest The authors have no conflict of interest and nothing to disclose.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

Informed consent Not applicable.

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