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Genomic and transcriptomic approaches to understand *Passiflora* physiology and to contribute to passionfruit breeding

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Abstract With more than 500 species, most of them occurring in the Neotropical region, the genus *Passiflora* is of key importance when one considers exploring biodiversity for food and medicinal purposes. Plants from the genus *Passiflora* produce fruits with economical importance, the passionfruits. Passionfruit breeding programs are leaving their infancy and molecular markers are increasingly being used to assist selection for desirable traits in novel passionfruit commercial varieties. However, the molecular and genetic basis of many of the selected characters affecting passionfruit production and quality cannot be studied in model species such as *Arabidopsis thaliana*, due to developmental particularities of *Passiflora* species. Therefore, in this review we comment on the development of genetic, genomic and transcriptomic tools that are recently becoming available for passionfruits.

Keywords Passionfruit · *Passiflora* · Transcriptomics · Reproductive development · Gene expression

1 Introduction

Passionfruit is the common name for many species belonging to the genus *Passiflora*. This genus is the largest one of the Passifloraceae family that encompasses more than 500 wild species distributed especially in the Neotropical region (Ulmer and MacDougal 2004). The commercial production of passionfruits is an activity of crucial economical importance for Brazil, mainly based on the yellow or sour passionfruit, *Passiflora edulis*. With around 50 thousand hectares producing around one million tons of fruits per year, Brazil is the largest passionfruit producer in the world, but it is also the highest consumer, absorbing near all its own production of fresh fruits, leaving insignificant amounts for export (IBGE 2017).

Passionfruit productivity in Brazil is very low and has stagnated around 14 ton per hectare for over a decade (Gonçalves and Souza 2006; Cavichioli et al. 2018). Nonetheless, there is a large discrepancy in productivity between areas where technology is incorporated by producers (more than 20 ton per hectare in Southeast Brazil) and areas where there is

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less investment in the orchards (around 11 ton per hectare in Northeast Brazil) (Gonçalves and Souza 2006; Cavichioli et al. 2018). Among the technologies employed for successful passionfruit commercial production is obviously genetic breeding for the development of improved varieties. Passionfruit breeders traditionally look after genotypes that produce more and larger fruits, but also that are resistant and/or tolerant to soil pathogens, viruses and abiotic stresses such as drought (Cerqueira-Silva et al. 2014; Freitas et al. 2016; Rosado et al. 2017). But more recently, there is a quest for products of aggregated valued such as fruits with different colors and flavors, ornamental varieties and varieties developed for the production of medicinal or cosmetic extracts (Abreu et al. 2009; Cavichioli et al. 2018; Cazarin 2013; Cerqueira-Silva et al. 2014; Giovannini et al. 2012; Santos et al. 2012). Therefore, understanding the genetic and molecular mechanisms that regulate these traits is not just a fascinating question of basic passionfruit physiology, but will also help to understand how these traits might be selected during passionfruit breeding and may also have important implications for the rational improvement of passionfruit quality and productivity.

2 The origin of passionfruits

The genus *Passiflora* L. is the largest of the Passifloraceae family, and the majority of these are herbaceous lianas but some passionfruits are also produced by shrubs and trees. Killip (1938) and MacDougal (1994) described a high morphological diversity among species, especially when flower structures are concerned, as *Passiflora* flowers display a wide variation in size and color, with synapomorphic structures such as the corona and the androgynophore (Fig. 1). Coevolution with pollinators has been suggested as an explanation for this observed diversity (MacDougal 1994).

Although early works suggested that the genus *Passiflora* should be divided into 22 or 23 subgenera (Killip 1938; Escobar 1989), there is a general agreement that only four subgenera (*Passiflora, Decaloba, Astrophea* and *Deidamioides*) are supported, as suggested by Feuillet and MacDougal (2004) and corroborated by more recent morphological, molecular and ecological data (Hansen et al. 2006; Muschner et al. 2003, 2012; Yotoko et al. 2011). The first molecular phylogeny of *Passiflora*, by Muschner et al. (2003), used plastidial and nuclear genome markers and clearly defined three major clades (*Passiflora, Decaloba, Astrophea*) with the fourth one (*Deidamioides*) less defined due to the small number of species classified in it. Accordingly, the available cytogenetic information on passion fruit species describes four different chromosome numbers distributed among the four subgenera of *Passiflora*: $x = 6$ in *Decaloba*, $x = 9$ and $x = 10$ or 11 in *Passiflora*, $x = 10$ in *Astrophaea*, and $x = 12$ in *Deidamiodes* (Melo et al. 2001; Melo and Guerra 2003). Although there is no agreement on the estimation of the *Passiflora* basic number of chromosomes, studies have suggested 6 or 12 as the basic number (Hansen et al. 2006; Melo et al. 2001). Although some species might be considered tetra- hexa- or even octoploid (Hansen et al. 2006; Melo et al. 2001), most of *Passiflora* species are considered diploid, with $2n = 12$, 18, or 20 chromosomes. The most commercially important species, such as *P. alata* and *P. edulis*, are diploid ($2n = 18$), which generally allows interspecific hybrids to be obtained for genetic breeding purposes (Abreu et al. 2009; Santos et al. 2012; Souza et al. 2008).

Although divergence time studies suggest a post-Gondwanic origin of the Passifloraceae (Muschner et al. 2012), they also indicated that when *Passiflora* ancestors arrived in Central America they diversified quickly from there. The very ancient (~ 40 Mya) separation of *Astrophea* from the other *Passiflora* clades indicates why this subgenus contains species that present the most unusual morphological traits within *Passiflora*. Nonetheless the diversification age within subgenus *Passiflora* (~ 16.8 Mya) was much more recent than the diversification in *Decaloba* (~ 29 Mya; Muschner et al. 2012). Most of the cultivated passionfruit species are not considered completely domesticated, although the first reports of passionfruit cultivation (mostly for ornamental purposes) date back from the XVII century when living plants were taken from central America to Europe (Ulmer and MacDougal 2004). The *Passiflora* species that is mostly cultivated world-wide nowadays is certainly *P. edulis*, wich is a semi-woody perennial climbing vine producing round to oval fruits containing hundreds of seeds, each surrounded by an aromatic juicy aril. Two major varieties, with purple (*P. edulis*

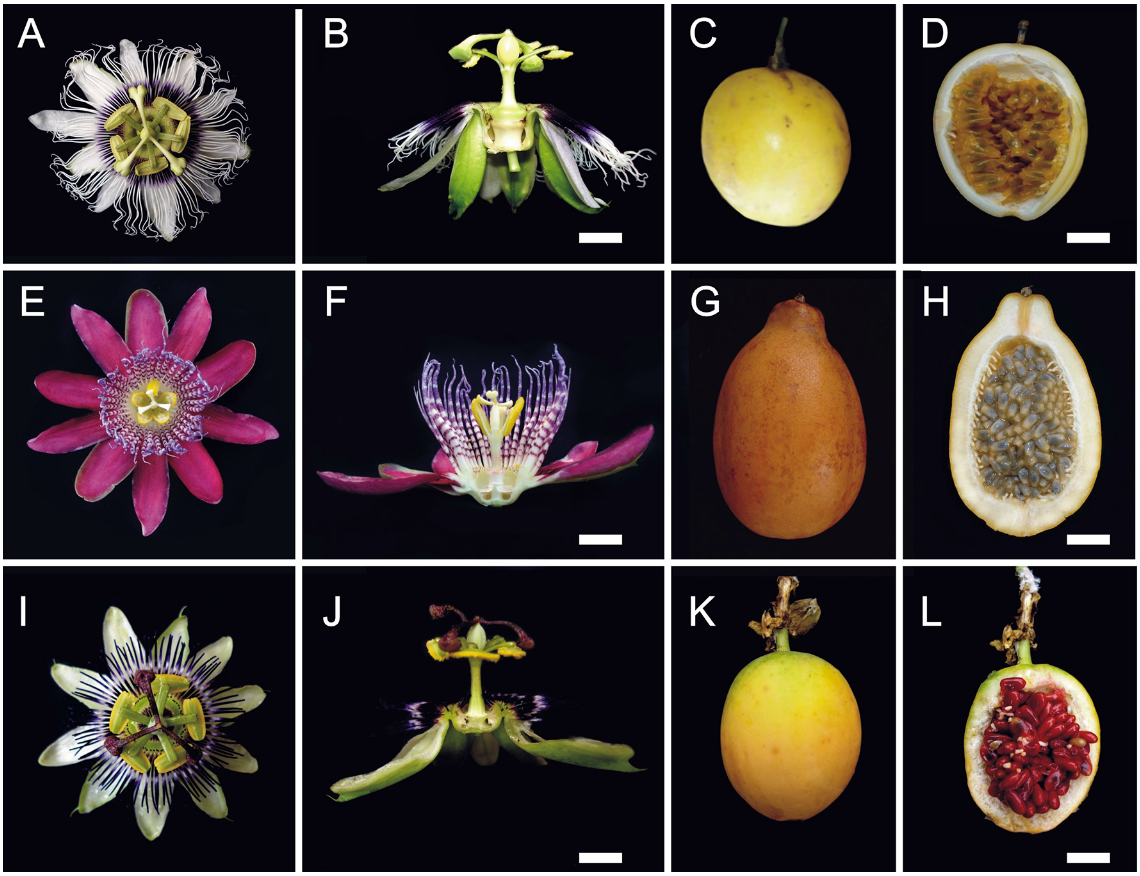

Fig. 1 Frontal view (**a**, **e**, **i**) and longitudinal sections (**b**, **f**, **j**) of flowers at anthesis of *Passiflora edulis* (**a**, **b**), *P. alata* (**e**, **f**) and *P. caerulea* (**i**, **j**). External aspect (**c**, **g**, **k**) and longitudinal sections (**d**, **h**, **l**) of mature fruits of *P. edulis* (**c**, **d**), *P. alata* (**g**, **h**) and *P. caerulea* (**k**, **l**). Bars: **a**, **b**, **e** and **f**: 1.5 cm; **c** and **d**: 2.5 cm; **g** and **h**: 3.5 cm; **i** and **j**: 1 cm; **k** and **l**: 2 cm

Sims f. edulis) and yellow (*P. edulis Sims f. flavicarpa* Deg.) fruits, are grown mainly for fresh fruit and/or juice production, in mild subtropical (purple variety) to warm tropical (yellow variety) climates (Menzel et al. 1987). The purple variety occurs wildly in southeast Brazil, Paraguay and northern Argentina, but the origin of the yellow variety (most commonly cultivated in Brazil) is unclear. Hybrids of these two varieties are commercially grown in Hawaii and Israel (Nave et al. 2010).

3 Approaches and tools used to study important passionfruit traits

From a commercial point of view, there are several very important passionfruit traits, depending on the purpose of cultivation. These traits include plant architecture, flower/fruit development, fruit seasonality and resistance to biotic and abiotic stresses (Cerqueira-Silva et al. 2014; Freitas et al. 2016; Rosado et al. 2017; Scorza et al. 2017). But let us not forget that *Passiflora* species are also highly valued for their use as ornamental and medicinal (or cosmetic) plants (Abreu et al. 2009; Cavichioli et al. 2018; Cazarin 2013; Cerqueira-Silva et al. 2014; Giovannini et al. 2012; Santos et al. 2012). During the past decade, molecular and genetic approaches started to be used to identify and functionally characterize genes and gene networks associated with important passionfruit traits (Aizza and Dornelas 2011; Rocha et al. 2015, 2016; Rosa et al. 2013a, b, c). However, until early 2000, very little information was available on gene expression in passionfruits. The very first report on gene cloning and gene expression analysis in passionfruits is the work of Mita et al. (1998) that studied fruit ripening processes and the influence of ethylene and the involvement of genes related to its biosynthesis and perception. The findings of Mita et al. (1998) could have biotechnological interest since manipulation of ethylene biosynthesis might be of interest to control fruit ripening. This is probably the only reported study on any molecular aspect of fruit development in *Passiflora*, although recently some groups started to be interested on the molecular basis of aril development (Silveira et al. 2016). More recently, a thorough physiological work was performed to clearly establish the role for etilene was established during the ripening process of passionfruits (Goldenberg et al. 2012) and the early molecular work by Mita et al. (1998) might contribute to elucidate the molecular aspects of this process.

Nonetheless, fruit production starts with the transition of juvenile plants to the reproductive stage followed by successful flowering. There is a great interest in understanding the molecular basis of *Passiflora* reproductive development, as the manipulation of flowering pathways might allow controlling fruit production. The control of flowering in *Passiflora* is based on the interplay between photoperiod and the balance between biosynthesis and perception of gibberellins and cytokinins (Chayut et al. 2014; Cutri et al. 2013; Nave et al. 2010; Sobol et al. 2014). Indeed, it has been shown that doubling the number of fruits per node might be a simple matter of increasing the production of cytokinins by the adult plant (Cutri et al. 2013). The molecular pathways involved in the interplay between photoperiod and hormonal balance is a key issue to be explored by *Passiflora* molecular biology studies in the near future as the outcome of this interplay, in model plants such as *Arabidopsis thaliana*, is the activation of inflorescence and flower meristem identity genes (see the review by Bloomer and Dean 2018). Regrettably, most of what is known of the molecular regulation of arabidopsis flowering might only partially be applicable to *Passiflora*. The study of the flowering processes in passionfruits is complicated by the shared common origins of flowers and tendrils (Cutri et al. 2013; Nave et al. 2010). Actually, *Passiflora* tendrils are thought to be modified flowers or inflorescences (Krosnick and Freudenstein 2005). Recent molecular characterization of floral development in *Passiflora* provided new molecular evidence that tendrils are indeed part of the *Passiflora* inflorescence and express putative orthologs of the *Arabidopsis APETALA1*, and *FRUITFULL* genes (Scorza et al. 2017). Besides pointing to the convergence of similar developmental processes involving the recruitment of genes related to flower identity in the origin of tendrils, this work clearly indicates that molecular resources are urgently needed in *Passiflora* as the traditional candidate gene approach using comparisons to plant models such as arabidopsis might not be useful for many passionfruit important traits.

On the other hand, very little is known about how passionfruits cope with abiotic and biotic stresses. Although some potyvirus and some aphid-borne mosaic viruses have been isolated and shown to affect passionfruit production (Barros et al. 2011; Chen et al. 2018; Yang et al. 2018), nothing is known about the molecular or physiological mechanisms involved. The key publication by Lopes et al. (2006) mapped resistance genes to *Xanthomonas axonopodis* pv. *passiflorae* in yellow passionfruit. They found quantitative resistance loci (QRLs) which explained a great deal of the total phenotypic variation. This work pointed to the importance of fine-tuning genetic mapping data for marker-assisted selection in passionfruit breeding programs. Additionally, Abreu and Aragão (2007) cloned the *PeMIPS1* gene from *P. edulis*, that is supposed to code for an enzyme involved in the biosynthesis of all inositol-containing compounds, and showed that it is differentially regulated under cold and heat stress, presenting a light-responsive transcription, indicating that this gene might be involved with environmental stress response.

4 Use of molecular markers as tools for *Passiflora* breeding and for the study of genotypic diversity

The first Passiflora linkage maps were available in the early 2000s. The aim of the groups that developed these maps was to locate or tag genomic regions associated with important passionfruit traits, most of the time quantitative trait loci (QTLs). Nonetheless, linkage analysis in passionfruit has been complicated by the difficulty of obtaining inbred lines, due to self-incompatibility and high heterozygosity (Cerqueira-Silva et al. 2014). The early passionfruit linkage maps were based on the characterization of a segregant population of *P. edulis* using random amplified polimofic DNA (RAPD) markers (Carneiro et al.

2002). Later, the use of amplified fragment length polymorphism (AFLP) markers, allowed the detection of QRLs potentially related to passionfruit genes conferring resistance to *Xanthomonas* (Lopes et al. 2006). One of the first steps to produce higher quality molecular marker-based maps for passionfruits was the introduction of microsatellite markers for map construction in addition to AFLP (Cazé et al. 2012; Cerqueira-Silva et al. 2012; Padua et al. 2005; Oliveira et al. 2005). This allowed the building of a single *P. edulis* integrated and highly saturated map that lead to the identification of the first QTLs in passionfruit (Oliveira et al. 2008). It did not take too long for other passionfruit species, such as *P. alata* to have its integrated genetic map (Penha et al. 2013; Pereira et al. 2013). This map also included the use of single sequence repeats (SSRs) and, for the first time, single nucleotide polimorphisms (SNPs) with potential practical application were detected in passionfruits (Costa et al. 2017; Pereira et al. 2017).

Recently, research dedicated to the construction of the first passionfruit physical map was published (Santos et al. 2014), that lead to the sequencing of the first gene-rich regions of a commercially important passionfruit species (Munhoz et al. 2018). Together with data on expressed sequence tags (ESTs) that first approached transcriptomics in *Passiflora* (Cutri and Dornelas 2012), the genomic age has clearly begun for passionfruits.

5 Transcriptomics and genomics as tools for the study of Passiflora biology and for *Passiflora* breeding

Studies dealing with transcriptomic arrived first in passionfruit literature, before actual genomic analysis. Cutri and Dornelas (2012) used the analysis of expressed sequence tags (ESTs) as an approach for the characterization of genes expressed during the reproductive development of *P. edulis* and *P. suberosa*. Combining both species, 10,272 assembled *Passiflora* sequences, assumed as proxy for representative gene (cDNA) sequences, were obtained. Among these sequences, many showed significant similarity to genes known to be involved in reproductive development in model plants (Cutri and Dornelas 2012). RT-PCR and in situ hybridization were used to confirm the expression patterns of some of the sequences during *Passiflora* flower development. Among these sequences there are *P. edulis* putative orthologs to the *Arabidopsis* KANADI and YABBY genes. In *Arabidopsis*, the YABBY and KANADI genes interact to establish and maintain the abaxial-adaxial polarity of all plant organs (Bowman 2000; Eshed et al. 2001). Manipulation of these gene expression patterns might alter organ size, including fruit size (Bowman 2000; Eshed et al. 2001). A second group of conserved genes that were found to express during *P. edulis* flower development belong to the now classical ABC model (Coen and Meyerowitz 1991). Putative *Passiflora* orthologs to the *Arabidopsis* C-class AGAMOUS gene and B-class PISTILLATA genes were found to be expressed in restricted domains of the floral whorls and were linked to the differentiation of the Passifloraceae-specific floral organ, the corona (Hemingway et al. 2011; Scorza et al. 2017). Understanding how floral morphology affects pollination might lead to knowledge that would allow incrementing pollination efficiency and thus, fruit formation. Additionally, putative orthologs to genes related to meristem behavior, important for the activity of the axillary meristems, were flowers are produced in *Passiflora*, were also found (Cutri and Dornelas 2012). These include members of the TCP gene family (see review by Martín-Trillo and Cubas 2010). It is known that members of the TCP family are closely linked to the process of formation of tendrils in Cucurbitaceae (Mizuno et al. 2015; Wang et al. 2015).

On the other hand, the use of suppression subtractive hybridization allowed Munhoz et al. (2015) to construct two cDNA libraries enriched for transcripts induced and repressed by the interaction of *P. edulis* with the passionfruit pathogen *Xanthomonas axonopodis*. Sixty-three out of the total of 998 obtained transcripts were similar to *Arabidopsis* defense-related proteins. From these, 76% (48/63) had their expression profiles changed in response to the pathogen as demonstrated by quantitative PCR data. The gene that responded most strongly to the pathogen attack encodes a lipoxygenase 2 (Munhoz et al. 2015). The detection of these novel sequences can contribute to the development of gene-based markers for important agronomic traits as well as to the establishment of genomic tools to study the naturally occurring diversity among *Passiflora* species.

The seminal work of Santos et al. (2014) provides an initial overview of the *P. edulis* genome using

BAC-end sequence (BES) data. The 'IAPAR-123' accession was used as a donor of genomic DNA to construct the BAC library. Around 9.6% of all BES were found to have high levels of similarity to plant genes and identities could be assigned to 940 of all the analyzed sequences. The results of comparative mapping between the BES of *P. edulis* and the reference genomes of *Arabidopsis thaliana*, *Populus trichocarpa* and *Vitis vinifera* were used to choose BAC clones for full sequencing (Munhoz et al. 2018). Over 100 large-inserts were completely sequenced and structural sequence annotation resulted in the prediction of about 1900 genes. Most of them were annotated as coding proteins involved in metabolic and cellular processes and biochemically involved in binding and catalytic activity (Munhoz et al. 2018). Noteworthy, almost all of the predicted encoded proteins showed sthe highest levels of similarity to proteins from *Jatropha curcas*, *Populus trichocarpa*, *Populus euphratica* and *Ricinus communis*. The authors mention that these results were expected, since all these represent species belonging to the Malpighiales order for which fully sequenced genomes are available and they are phylogenetically close to *P. edulis*. Accordingly, there is a high degree of comparative microsynteny between *P. edulis* and both *P. trichocarpa* and *M. esculenta*. These results are the foundation of a novel era of comparative genomics in *Passiflora* but further studies are still necessary to access the abundance of repetitive DNA associated with gene-poor regions and the annotation of a larger number of Passiflora genes, since the (already large) number of available annotated *P. edulis* genes represent only 1% of the expected number of genes in the genome.

Finally the complete *P. edulis* chloroplast genome became available recently (Cauz-Santos et al. 2017). It is characterized by the presence of two copies of an inverted repeat sequence, each separating a two single copy regions of 13,378 bp and 85,720 bp. The annotation of the *P. edulis* chloroplast genome identified 105 unique genes, including 30 tRNAs, 4 rRNAs, and 71 protein coding genes (Cauz-Santos et al. 2017). These sequences might be useful as genetic markers, taking into account that chloroplast inheritance in *Passiflora* might be either paternal or maternal, depending on the species (Muschner et al. 2006).

6 Conclusions and future perspectives

The last decade has presented the *Passiflora* literature with a large number of papers that indicate that passionfruit species have entered the "omics" era where large amounts of gene and protein sequence are becoming available for functional studies and/or for their potential use as genetic markers to assist breeding programs. It will be of great interest that we will expect the unfolding of novel available genomic resources for *Passiflora* species, such as the complete sequencing of the *P. organensis genome* (unpublished results from our group) the smallest *Passiflora* genome with around 300 Mbp (Yotoko et al. 2011). Also as the techniques for obtaining transgenic plants is already available for passionfruits (Manders et al. 1994; Monteiro-Hara et al. 2011; Trevisan et al. 2006) genetic manipulation of candidate genes is already possible.

The challenge ahead is that many of the traits that might be interesting for passionfruit breeding will have to be studied in *Passiflora* species and will have limited profit from studies conducted in model plants such as *Arabidopsis*. This is because contrary to what is observed in *Arabidopsis*, passionfruits are semi-perenial plants and flowers (and thus fruits) are produced by the axillary meristems and not by the conversion of the apical shoot meristem into an inflorescence. Additionally, *Arabidopsis* does not form tendrils (which have a shared morphological origin with flowers in passionfruits) and produces dried dehiscent fruits (siliques) opposed to *Passiflora* species that produce (at least in the species of commercial interest) indehiscent fruits whose seeds are involved with a juice sac, the aril. The aril being a structure that is also absent in *Arabidopsis*. As for reasons of space restrictions, we intentionally neglected aspects of *Passiflora* biology related to the production of medicinal compounds (most of them glycosides, alakloids, flavonoids and phenolic compounds, Cazarin 2013) that, although some of them might also be present in *Arabidopsis* and thus share a conserved biosynthetic pathway, the final steps in their biosynthesis might involve proteins present exclusively in the *Passiflora* genome, as it was observed for compounds involved in the composition of rose and citrus scents (Amrad et al. 2016).

Based on the observations above, it has become clear that the model species suited for the study of passionfruit physiology and the source of gain of knowledge that will benefit genetic breeding programs are not those already available such as *Arabidopsis*, but must come from the genus *Passiflora* itself. Therefore this is the reason why passionfruit genomic resources will continue to be prized and will largely contribute to the understanding of Passiflora physiology and to breeding programs in the decades to come.

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