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Review

The dynamic genetic-hormonal regulatory network controlling the trichome development in leaves

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Abstract: Plant trichomes are specialized unicellular structures that originate and project from above ground epidermal tissues on the surfaces of leaves, petals, stems, petioles, peduncles, and seed coats depending on species. Trichomes (also called 'hairs') play well-recognized roles in defense against insect herbivores, both as a physical barrier that obstructs herbivore movement and by mediating chemical defenses. By virtue of their physical properties (size, density), trichome hairs can directly operate to protect buds of plants from insect damage, reduce leaf temperature, increase light reflectance, prevent water loss, and decrease leaf abrasion. Great variety of trichomes and their accessibility makes them a useful model for studying the molecular processes of cell fate determination, cell cycle control and cellular morphogenesis. In leaves, the developmental control of the trichomatous complement has highlighted a regulatory network based on four fundamental elements: (i) genes that activate and/or modify the normal cell cycle of epidermal cells (i.e. endoreduplication cycles); (ii) transcription factors that create activator/repressor complexes with a central role in determining cell fate, initiation and differentiation of an epidermal cell in trichome; (iii) evidences that point out the interplay of the aforesaid complexes with various phytohormones; (iv) epigenetic mechanisms involved in trichome development. Here, we describe trichome development in leaves, commonly subjected to environmental injury, and where most genetic regulators have been characterized.

Keywords: trichomes; transcription factors; hormones; endoreduplication cycle; epigenetic mechanisms

1. Introduction

The epidermis is the superficial coating layer that wraps the leaves and the primary body of the stem and it is in direct contact with the atmosphere, therefore a protective barrier against abiotic and biotic factors. The epidermis is not a homogeneous tissue: it is made up of epidermal cells properly so called and by annexed (or specialized) cells such as stomata and trichomes or hairs [1-7].

Trichomes are defined as epidermal projections consisting of single or groups of cells with different shapes, sizes, structures and functions. Located on the surface of any part of the plant, they can be persistent or ephemeral, alive or dead. Moreover, trichomes can be unicellular, multicellular, simple or branched, starry, squamiform or glandular [1,3,7,8]. Every single hair originates from an epidermal cell (initial cell): in some cases, the latter forms, by distension, a long extroflexion, generating a unicellular hair. When instead the mother cell undergoes repeated divisions, a pluricellular hair is originated. Often there are more types of hairs in the same organism, and the term "trichome complement" is referred to as the set of all the hairs, present on the surface of the plant, with different characteristics and functions [5,9]. The protective function of the hair is very common. Hairs with this role are generally dead and filled with air: this is what gives shine and whitish color, a sign of the reflection of light of which they are capable; in this way, they ensure an

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effective protection against solar radiation and preserve the plant (especially the leaves) from excessive water loss through transpiration. More rarely, in plants grown in humid environments, the epidermis is provided with alive hair. In this instance, the hairs enhance the transpiring surface of the leaf and, consequently, the leaf transpiration is increased. In addition to the protective role, other functions of trichomes can be considered, including those of support, absorption, secretion, dissemination and perception of external stimuli [8,9]. Notably, glandular trichomes are metabolically highly diverse, and they synthesize, store and release secondary metabolites such as terpenoids, flavonoids and phenylpropanoid, alkaloid, acyl sugars and defensive proteins [10-15]. Many of these chemicals are thought to function in defense against herbivores and arthropods, and they have received extended research interest because of the potential use of these compounds in pharmaceutical and nutraceutical applications [16-20]. Trichomes defend the plant not only against animal damage but also from other external factors such as an excess of UV light or high temperatures [21-23]. Peculiar structures are the trichomes in root hemiparasites from the rhinanthoid clade of Orobanchaceae that possess metabolically active glandular hairs that have been suggested to function as hydathode trichomes actively secreting water, a process that may help in the acquisition of nutrients from the xylem of host plants [24]. Cotton fibers are trichomes of cotton ovules, which may have aided in precultivation seed dispersal [25].

Notably, trichomes are important markers of plant developmental stages and therefore, with a relevant role for researches on heterochronic processes [26]. For example, in the model plant *Arabidopsis thaliana*, each developmental phase is characterized by well-defined morphological traits such as alterations in leaf size and shape (heteroblasty), the expression of genes linked to juvenility and the presence/absence of unicellular trichomes on the abaxial leaf surface [27,28]. In *Arabidopsis*, mature trichomes are localized on the surface of leaves, stems and sepals. On mature leaves of trichomes are branched with a single stalk sustaining three spikes [29].

Usually, plant embryos are devoted of trichomes and at seedling stage, cotyledons and hypocotyl of *Arabidopsis* are glabrous because the first trichomes differentiate on adaxial surface of the first pair of leaves [29]. Trichome precursor cells become visible in leaf primordia of 100 μm in length and during very early stage; the initiating hairs are four epidermal cells separately [1]. Initiation of trichomes is regularly spaced and these do not belong to the same cell lineage [30]. The pattern of initiation is influenced by a field of inhibition originating within each developing trichome and extending two-three cells beyond the hair [1,30]. However, the establishment of trichome pattern *in vivo* is not an obvious phenomenon [8,31].

Correlations between leaf shape and the numbers and size of trichomes were observed in some species such as *Begonia dregei*, where, significant correlations between the shapes of leaves and the presence, number, and size of trichomes among populations was see [32]. Furthermore, in hybrid plants, leaves characterized by deeply incisions had larger numbers of longer trichomes at the lobes [32]. Mutants of *Arabidopsis* for leaf shape can also be affected in the trichome branching pattern [1]. Finally, irregular pattern of trichome differentiation can be observed in specific leaf areas as in galls induced by insect colonization [33].

2. Role of genes in the development of trichomes overall in model species

The regulatory genes involved in the development of leaf trichomes have been mostly studied in *Arabidopsis* because of the availability of several mutants with defects in initiation and development of these structures. In this species, trichomes have a typical unicellular structure and their origin from the epidermis comprises three successive phases: determination of cell fate, specification and morphogenesis [1,8,9,34]. While the other cells belonging to the epidermis continue to divide, the trichomatous cells enter a phase characterized by one to four cycles of endoreduplication, reaching a mean DNA (C) content equal to 32C (see multicellular trichomes). The origin of the trichomes begins at the base of the young leaf, a phase in which all the cells are potentially competent to develop trichomes. However, as above reported, the epidermal cells that generate the leaf hairs are arranged at regular intervals of distance from each other [8]. Usually, in wild type *Arabidopsis*, no trichome clusters have been detected on the leaf epidermis. This requires a

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mechanism able to regulate the spatial arrangement of the hairs on the leaf surface [8,31]. A similar phenomenon is observed in the development of stomata [6].

More than 70 genes control trichome development in *Arabidopsis* [5,7-9,34]. It has been hypothesized that the initiation of the trichomes on the epidermis is based on a mechanism constituted by the activity of genes, which can be divided into two fundamental groups: positive and negative regulators [2,8,9,35]. In *Arabidopsis*, positive regulators include, *GLABROUS1* (*GL1*) and its homologous *WEREWOLF* (*WER*) encoding R2R3-MYB transcription factors (TFs) [36], the *GLABRA3* (*GL3*) gene encoding a basic Helix-Loop-Helix (bHLH) TF with the homologous gene *ENHANCER OF GLABRA3* (*EGL3*) [37-41], and the *TRANSPARENT TESTA GLABRA1* gene (*TTG1*) that encodes a protein containing a WD40 repeat (also known as WD or beta-transducin *repeats*) a short ~40 amino acid motifs, often terminating in a Trp-Asp (W-D) dipeptide [42-44]. *GL1* and *TTG1* appear to play an essential role for the initiation of the trichomes since the *gl1* and *ttg1* mutants exhibit almost a hairless phenotype, and in *gl1*, only a few trichomes are initiate at the leaf margin [1,2,38,45,46]. *GL1* and *TTG1* control the same process in trichome initiation [45,46], although *TTG1* is also involved in the regulation of flavonoid production [44]. In *ttg1* mutants, the anthocyanins that give a reddish color to parts of seedlings, stems, and leaves, particularly under stress conditions, are absent [47]. In addition, it is also absent the dense brown tannin produced by the inner layer of the seed coat [48]. A model based on the activation of epidermal cell to differentiate trichomes was analyzed by the depletion of *TTG1*. This model hypothesized that initially all epidermal cells expressed *TTG1* equally, but its level of expression drastically decreased in cells adjacent to those that will initiate trichome development [8,9]. Really, *TTG1* can move freely between young tissues and accumulate in cells containing high levels of *GL3*; therefore, cells with a high content of the GL3/TTG1 complex will be able to develop trichomes unlike neighboring cells in which TTG1 will be insufficient for the determination of the epidermal cell to differentiate leaf follicles [8]. Collectively these observations suggest that GL1 and TTG1 interact with GL3/EGL3 to form an activator trimeric complex MYB/bHLH/WD (MBW) [42,43,49-52]. This regulatory "pool" stimulates epidermal cells to differentiate into trichomatous cells (Figure 1), promoting the expression of the activators *GLABRA2* (*GL2*) and *TRANSPARENT TESTA GLABRA2* (*TTG2*) that encode for a "Homeo Domain-Leucine Zipper" (HD-Zip) and a WRKY TF, respectively [5,7-9,53-60].

The *gl2* mutant produces anomalous trichomes (not expanded and most unbranched), analogously a major effect of *ttg2* mutation on trichome development is to reduce or eliminate branching. These observations suggest that these genes are also required for the regulation of the trichome complexity [55]. Notably, in *Brassica napus*, four *BnaTTG2* genes rescue the phenotypes of *Arabidopsis ttg2* mutants [61]. The over-expression of *BnaA.TTG2.a.1* also enhanced the trichome numbers in both *Arabidopsis* and *B. napus* plants. Notably, the *BnaA.TTG2.a.1*-over-expressing plants of both species also showed an increased sensitivity to salt stress [61]. Moreover, in *Arabidopsis* plants under salt stress and over-expressing *BnaA.TTG2.a.1*, the endogenous indole-3-acetic acid (IAA) level was decreased, and the expression of *TRYPTOPHAN BIOSYNTHESIS 5* (*TRP5*) and *YUCCA2* (YUC2) genes, was down regulated. Therefore, Li et al. [61] suggested a new role for *Bna.TTG2* genes in salt stress responses and auxin metabolism. In the trichomatous cell, the MBW complex also stimulates the development of trichomes by activating the expression of *SIAMESE* (*SIM*) and *RETINOBLASTOMA RELATED1* (*RBR1*) as cell cycle regulators (Figure 1). See after the regulation of the cell cycle and the trichome complexity.

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Acquisition of trichome competence in *Arabidopsis* epidermal cell

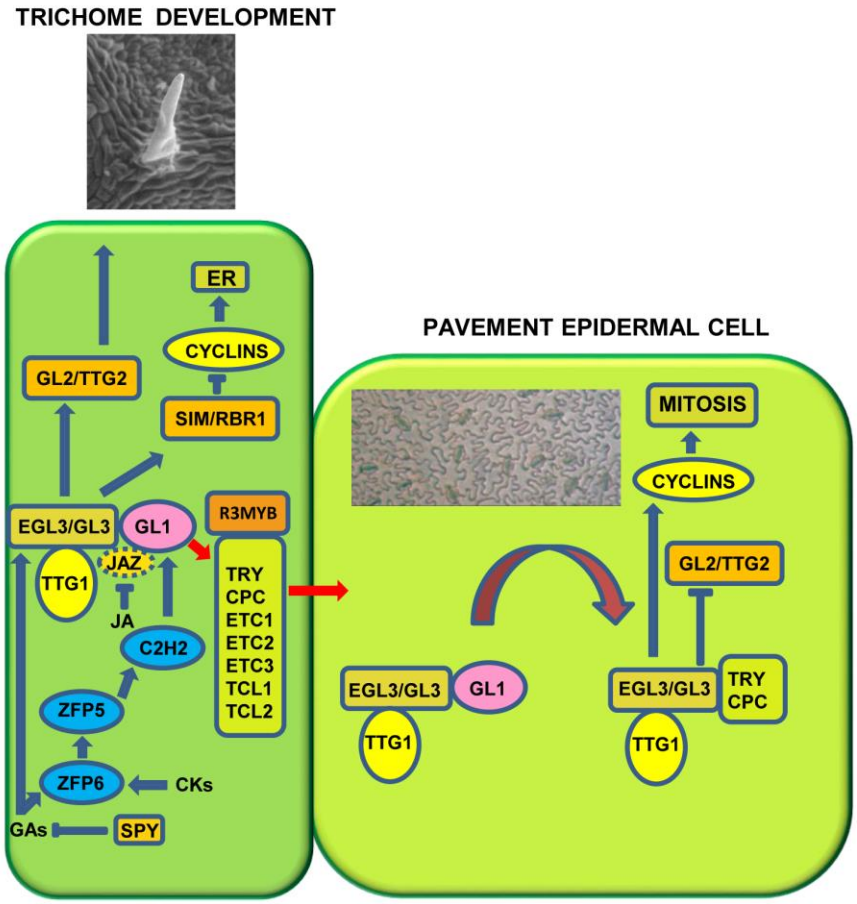

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Figure 1. A simplified model in the acquisition of the competence of epidermal pavement cells to become trichomes in the model species *Arabidopsis thaliana*. In epidermal pavement cell, GLABRA3 (GL3) physically interacts with GLABRA1 (GL1) and TRASPARENT TESTA GLABRA1 (TTG1), creating a trimeric MYB/bHLH/WD (MBW) activator complex. TTG1 acts upstream of GL3 and GL1, activating their transcription. Gibberellins (GAs), cytokinins (CKs) and jasmonic acid (JA) participate positively in the control of trichome initiation: GAs activate the expression of Zing Finger Protein 6 (ZFP6), which in turn induces the expression of GLABROUS INFLORESCENCE STEMS (GIS) (C2H2) (blue arrows). At the same time, ZFP8 (C2H2) and GIS2 (C2H2) are activated by ZFP5 and CK (blue arrows). The transcription of GL1 is intensified by C2H2. The *SPINDLY* (*SPY*) gene inhibits the GA signal. The jasmonic acid (JA) regulates the formation of trichomes, working on the degradation of Jasmonate ZIM-domain (JAZ) proteins (blue dotted line), and therefore, this hormone inhibits the interaction between JAZ with GL1 and EGL3/GL3. The MBW complex stimulates the development of trichomes by activating the expression of its direct targets: SIAMESE (SIM) and RETINOBLASTOMA RELATED1 (RBR1) as cell cycle regulators, GL2 and TTG2 as transcription factors and (CAPRICE) CPC, (TRYPTICON) TRY, ENHANCER OF TRY AND CPC1 (ETC1), ETC2, ETC3, TRICHOMELESS1 (TCL1) and TCL2 as inhibitors. SIM and RBR1 promote the differentiation of epidermal cells into trichomes, through the repression of the expression of the CYCLINS (CYCD3; 1 and CYCB1; 2) genes and inducing the transition from the mitosis to the endoreduplication cycle (ER). The inhibitors can move in the neighboring cells and replace GL1 in the MBW complex and form a repressor complex, this determines the activity of CYCD3; 1 and CYCB1; 2 and the initiation of the mitotic process. ER: endoreduplication cycle.

Dynamic regulatory network controlling trichome initiation and development involve other TFs. For example, a positive regulator in hair development is *MYB82* given that its over-expression determines the development of branched trichomes [62]. In addition, *MYB82*, activated by the *GL1* promoter, was able to complement the *gl1* phenotype, suggesting that *MYB82* was functionally redundant to *GL1* [62]. Notably, the second intron of *MYB82* includes regulatory motifs for both the temporal and spatial regulation of *GL3* [62,63]. Really, analogously to *GL1*, *MYB82* also interact with *GL3*, suggesting that in *Arabidopsis* MYB82 is likely incorporated into the activator MBW complex, playing a role in the regulation of trichome development [64,65].

The activity of additional genes is required in stage-specific phases of trichome differentiation. For example, *GLASSY HAIR* (*GLH*) genes of *Arabidopsis* are essential for the arrangement of surface papillae structures at late phases of trichome development [66]. Trichomes in *glh* mutants appeared transparent due to unhindered light transmission. In particular, seven different gene *loci* were identified. Two *loci* matched *TRICHOME BIREFRINGENCE* (*TBR*) and *NOECK* (*NOK*) genes [67-69]. *NOK* belongs to the *MIXTA* subfamily of *MYB* genes [70], which in *Arabidopsis* repress branching of trichomes [67]. Both of *glh2* and *glh4* trichomes showed a significant reduction in cellulose. In addition to the glassy trichome phenotype, the *glh6* mutant displayed defects in leaf cuticular wax [66]. Lastly, *glh1* and *glh3* trichomes showed reduced papillae formation. Based on these observations, Suo et al. [66] suggested that the *GLH1* and *GLH3* genes could have specific functions in trichome papillae formation, whereas *GLH2*, *GLH4*, and *GLH6* genes are likely required in deposition of additional cell-wall components. *TBR* belongs to the *TRICHOME BIREFRINGENCE-Like* (*TBL*) gene family. Members of the TBL protein family had been shown to affect pathogen resistance, freezing tolerance, and synthesis of secondary wall cellulose [68]. In trichome differentiation, the gene *TBR* plays a key role in the cellulose content but also regulates the trichome density on the epidermal surface [68,69].

Plant non-specific lipid transfer proteins (nsLTPs) belong to a large multigene family that possesses complex physiological functions such as the stabilization of membranes, cell wall organization, and signal transduction [71]. nsLTPs are also known to play important roles in resistance to biotic and abiotic stress, and in plant growth and development, such as sexual reproduction, seed development and germination [71]. Notably, in leaves of *Brassica napus*, over-expression of *BraLTP2* causes an increase in trichome number and an altered accumulation of secondary metabolites [72]. In tobacco, several *LTPs* that accumulated specifically in trichomes were

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identified [73]. Choi et al. [74] showed that tobacco *NtLTP1*, which was specifically expressed in long secretory glandular trichomes, plays a role in lipid secretion from trichome and in resistance to aphid infestation.

Negative regulators of trichome initiation and outgrowth consist of at least seven genes: *CAPRICE* (*CPC*), *TRIPTYCHON* (*TRY*), *ENHANCER OF TRY AND CPC1* (*ETC1*), *ETC2*, *ETC3*, *TRICHOMELESS1* (*TCL1*) and *TCL2* all of them coding for single-repeat (R), R3-MYB TFs [3,7,9,35,75-78]. A phylogenetic study places *TRY* and *ETC2* in one cluster and *CPC, TCL1, ETC1*, and *ETC3* in a separate cluster [79]. The seven genes share partially redundant functions in trichome and root hair formation. An over-expression of these TFs induces glabrous phenotypes but a single R3-MYB mutation leads to different phenotypes indicating that these genes do not have a fully redundant activity [9]. In fact, *cpc*, *etc2* and *etc3* mutants showed a greater density of trichomes; in leaves of *try* and *cpc;try* double mutants, a more clusters of trichomes were differentiate while *tcl1* and *tcl2* mutants did not exhibit variation in leaf density of trichomes but an increase of the trichomatous complement in their reproductive organs, stamens and inflorescences [23,35,75,76]. These results suggested that *TRY* was leading in controlling the formation of "clusters" of leaf trichomes, while *TCL1* and *TCL2* played a key role in the development of trichomes in organs of the inflorescence [8,9]. Therefore, although the functions of *CPC*, *TRY*, *ETC1*, *ETC2* and *ETC3* was partially redundant, their gene activity was prevalent in distinct spatial domains and provided actual evidence for the connection between gene expression and trichome spatial determination [5,8,9].

These inhibitors can move laterally in the epidermis between neighboring cells, competing with *GL1* and interacting with *GL3*/*EGL3*, thus inactivating the trimeric complex that becomes unable to trigger the expression of *GL2* and *TTG2* and then repress trichome initiation in adjacent cells [50,80]. In particular, GL1 and GL3 both contain a domain for DNA binding, and the alteration of these regions repress the expression of *GL2* (Figure 1). *TTG1* is able to activate the transcription of the complex *GL3*/*GL1*, demonstrating that *TTG1* operates upstream of these genes. *GL3* and *TTG2* are able to repress its expression while the inhibitory genes *TRY*, *CPC*, *ETC1* and *ETC3* are induced by the activators, so the activation of the *CPC* and *TRY* promoters needs a direct binding with GL3 [9,50,57,81-83]. In addition, TTG2 binds to W-boxes in a promoter fragment of *TRY*, and these W-boxes are essential for the rescue of the *try* mutant phenotype [83]. It was also showed that TTG2 alone is not able to activate *TRY* expression but increases the activation by *TTG1* and *GL3*. It was proposed that TTG2 enhances the activity of TTG1 and GL3 by forming a protein complex [83]. Moreover, *GL1* represses the activation of the *TRY* promoter by *GL3* and *TTG1*, and *TTG1* suppresses the activation of the *CPC* promoter by *GL1* and *GL3* [84]. Therefore, a regulatory loop involving a local autonomous circuit of multiple activators and repressors controls the expression of downstream gene targets and ultimately trichome formation [5,8,9]. The MBW activator complex induces the expression of genes encoding the repressors (TRY/CPC) which can move into neighboring cells to form a repressor complex (GL3/EGL3-CPC/TRY-TTG1) this also promotes the activity of cyclins and the activation of the mitotic process (Figure 1). Therefore, it is inhibited the function of the activators of trichome initiation [5,7-9].

Additionally genes, which extend the network involving positive and negative regulators of epidermal cell fate, trichome initiation and differentiation, have been discovered and several examples can be mentioned. For example, AtMYC1 bHLH TF of *Arabidopsis* was identified as direct targets of both *GL1* and *GL3* genes [85]. AtMYC1 could operate as a positive regulator of trichome initiation since trichome number is reduced in *atmyc1* mutant plants [85,86]. Notably, *GL3*/*EGL3* can replace *AtMYC1* activity, whereas *AtMYC1* cannot rescue *gl3* and *egl3* phenotypes, suggesting a redundant role but also a different function of these genes [86]. Expression analyses also showed that *AtMYC1* operated upstream of *GL2* [86]. In addition to GL3, AtMYC1 protein also interacts with most of the other patterning proteins including CPC, TRY, TTG1, GL1 and MYB23 [86-88]. However, in contrast to GL3 and EGL3, AtMYC1 protein appeared to be unable to form homo- or heterodimers with GL3/EGL3 [86]. Pesch et al. [89] also have showed that *GL2*, *TRY* and *CPC* expression patterns were unchanged in *atmyc1* mutants. Co-expression of *AtMYC1* with *TRY* or *CPC* leads to the

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recruitment of AtMYC1 into the nucleus, as well as to the transport of TRY/CPC from the cytoplasm into the nucleus. Therefore, Pesch et al. [89] have suggested that AtMYC1 inhibited the function of TRY/CPC.

In *Arabidopsis*, *CSN5a*, encoding COP9 signalosome subunit 5a, has been implicated in trichome production and the metabolism of various phenylpropanoid and carotenoid compounds as well as a glycoside of zeatin [90]. In particular, Wei et al. [90] have analyzed of a new *csn5a* mutant, *sk372*, characterized by enhanced anthocyanin accumulation as well as significantly reduced trichome density and distorted trichome morphology. The mutant phenotype were related to the enhanced *MYB75* and suppressed *GL2* activities as well as to modulation in the expression of genes associated with the MBW activator complex [90].

Notably, MADS box genes also appear to control trichome development. *AGAMOUS* (*AG*) suppress the formation of branched trichomes on carpel valves by controlling key regulatory genes [91]. In particular, it was demonstrated that *AG* regulates cytokinin responses and interacts with the organ polarity gene *KANADI1* to suppress trichome initiation in gynoecia [92].

The *TOO MANY MOUTH* (*TMM*) gene is involved in the regulation of stomata distribution and patterning [6]. However, Yan et al. [93] have revealed a new function of *TMM* in trichome development in *Arabidopsis* plants. In particular, in *Arabidopsis* over-expressing *TMM* the number of trichomes on leaves was significantly decreased and many of the trichomes had abnormal branches [93], which partially mimicked the mutants with fewer or loss of trichomes such as *gl1* and *ttg1* [1,94], and mutants with reduced branches, such as *stichel*, *angustifolia*, and *zwichel* [95]. Moreover, the reduction of trichome density was more obvious in reproductive than in vegetative stage. This suggested that *TMM* might have more important involvement in advanced stages of plant development [93].

The MBW activator complex controlling trichome initiation also positively regulates the late structural genes in the *Arabidopsis* flavonoid biosynthetic pathway that involves MYB75/90/113/114, GL3/EGL3/TT8 and TTG1 [38,43,87,96-98]. On the other hand, TRY and CPC compete with R2R3-MYBs for binding with the bHLH factors and alter the MBW complex, thus repressing at the same time trichome development and anthocyanin synthesis [99,100].

Patra et al. [101] proved that the Ubiquitin/26S Proteasome System (UPS) regulates post-translationally the MBW activator complex. The 26S proteasome is a multisubunit ATP-dependent protease complex crucial for regulated protein turnover in eukaryotes [102,103]. Conjugation of ubiquitin to proteolytic substrates marks them for degradation by the proteasome. The 26S proteasome is composed of two functionally distinct complexes, the 20S Core Protease (CP) and the 19S Regulatory Particle (RP) [102-104]. Patra et al. [101] have also showed that both GL3 and EGL3 were unstable and were targeted for UPS-dependent proteolysis. The UPS includes E1, E2 and E3 enzymes, whose combined actions are responsible for the conjugation of polyubiquitin chains that target proteins for proteolysis by the multi-subunit 26S proteasome [105-106]. Patra et al. [101] demonstrated that the proteasomal degradation of GL3 and EGL3 was mediated by E3 ubiquitin-protein ligase (UPL3). In addition, it was showed that mutation in the *gl3* locus negatively affects *UPL3* expression, whereas over-expression of *GL3* up-regulates it, suggesting the presence of a regulatory loop involving *GL3* and *UPL3* [101-107].

3. Gene and hormonal interaction in trichome development

Several studies show that the differentiation of the trichomes of plants is also regulated by the phytohormones, however, the ways in which they act are not fully known. The cytokinins (CKs) stimulate the formation of the trichomes overall on the inflorescences while the gibberellins (GAs) and jasmonic acid (JA) act synergistically on induction, number and density of the hairs on various organs [5,9,108-110]. Therefore, the three phytohormones act positively on the regulation of the growth of the trichomes; by contrast, the salicylic acid (SA) has a negative effect on trichome development [111]. For example, it was showed that exogenous treatments with GA on the hairless mutant, deficient in GAs, *gal-3*, stimulated the formation of trichomes, suggesting a positive action of GAs on the growth of leaf hairs [112]. In addition, Perazza et al. [113] showed that GAs promote

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the development of trichomes in *gl1* mutants by directly regulating the *GL1* gene. Furthermore, the transcription level of *GL3*, *TTG1* and *TRY* were also regulated by GAs. CKs and GAs also activate the expression of *GLABROUS INFLORESCENCE STEMS* (*GIS*), *GIS2* and *ZINC FINGER PROTEIN 8* (*ZFP8*), all coding for "zinc-finger" C2H2 TFs, which are supposed to control in concert, the transcription of *GL1* and *SIM* [114,115]. Another protein, ZFP5, through the GA signal, was able to activate and fine-tune the functions of *GIS*, *GIS2*, *ZFP8*, *GL1* and *GL3* and then to control the production of trichomes [116]. In particular, *GIS*, acting upstream of the MBW activator complex, promoted trichome initiation and outgrowth in response to GA signaling in *Arabidopsis* [114,117-121]. Over-expression of *GIS* triggers an increase of trichomes on inflorescence organs and other heterochronic phenotypes, while the loss of GIS function had opposite effects on trichome initiation. In fact, Gan et al. [117] demonstrated a decreased trichome production on inflorescence leaves, stem internodes, branches and flowers of the *gis* mutant. In addition, the *SPINDLY* (*SPY*) gene inhibits the GA signal [122-123], and *spy* mutants displayed an excessive number of trichomes. Gan et al. [117] have also showed that *GIS* operates upstream of *GL1* and downstream of *SPY*; furthermore, *GIS* is in contrast with the action carried out by the gene of the repressor of *GAI* [117]. New TFs that belong to the GIS clade, which may play redundant roles in integrating GA and CK signaling, such as *ZFP5*, *ZFP6* and *GIS3* trichome activators have been identified [118,119,121]. In *Arabidopsis*, like the phenotypes of mutants in any of the genes of the trichome MBW activator complex, loss of GIS-clade function decreases the trichome formation on the adaxial surface of rosette leaves and/or inflorescence organs. In addition, over-expression of any of these proteins generate a high density of trichomes [117-119,121,124]. It was showed that *GIS3* acts upstream of *GIS*, *GIS2*, *ZFP8* and the trichome initiation factors, GL1 and GL3, and it was suggested that *GIS* and *GIS2* were the direct target genes of GIS3 [118]. More recently, it was demonstrated that also in *Nicotiana benthamiana*, *NbGIS* was required in response to GA signal to control glandular trichome initiation [125]. In addition, *NbMYB123-like* regulated glandular trichome initiation in tobacco by acting downstream of *NbGIS* [125].

In *Arabidopsis*, the TRICHOME-RELATED PROTEIN (TRP) is a recently isolated TF that negatively regulates trichome initiation through GA signaling [126]. The *trp* mutant has an increased number of trichomes on flowers, cauline leaves, and inflorescence stems compared to normal plants. By contrast, plants over-expressing *TRP* exhibit fewer trichomes on cauline leaves and inflorescence stem because of exogenous GA treatments. It is likely that TRP operates upstream of the trichome initiation regulators repressing the binding of *ZFP5* to the *ZFP8* promoter [126].

TEMPRANILLO1 (TEM1) and TEM2 TFs, are two proteins belonging to the small plant-specific RELATED TO ABI3 AND VP1 (RAV) family, and initially identified as repressors of floral induction [127-128]. More recently, it was demonstrated that TEM1 and TEM2 repress trichome initiation by controlling GA accumulation and distribution in the leaf mesophyll as well as by integrating both GA- and CK-dependent regulatory pathways, which in turn negatively affect trichome formation in all epidermal tissues of *Arabidopsis* [129]. Matías-Hernández et al. [129] showed that TEM1 and TEM2 operate redundantly to repress the transcription of most essential positive epidermal regulators of trichome initiation and growth. In particular, both TEM1 and TEM2 repress *GL2* owing to the fact that both GA- and CK-dependent trichome pathways converge in its activation. In addition, since *tem2-2* mutant plants produce more trichomes than *tem1-1* and normal plants, Matías-Hernández et al. [129] have suggested that *TEM2* may play a more significant role in trichome initiation in comparison to the activity of *TEM1*.

In *Arabidopsis*, a subunit of the ubiquitin-mediated 26S proteasome (RPN1a), involved in the development of branched trichomes, interacts with both GAs and CKs [5]. Mutations in *RPN1a* generate more branched trichomes on leaves [130]. In the *rpn1a* mutant plants, the transcription levels of *ZFP5*, *ZFP6*, *GIS*, *GL1*, *GL2*, *GL3*, *TTG1* and *MYB23*, which promote trichome initiation, are up regulated. In addition, the expression of *FURCA4* (FRC4), which is responsible for increased trichome branching, is also enhanced in the *rpn1a* mutant in comparison to wild type [116,130]. The mRNA expression level of *RPN1a* is significantly repressed by GA and CK treatments. It was

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suggested that *RPN1a* could be involved in trichome development through the GA and CK signalling pathways [130].

The 6-benzylaminopurine (BAP, CK) is a positive regulator of trichome development since *Arabidopsis* BAP-treated plants develop more trichomes on leaf; however, the trichomes are shorter and nuclear DNA content is less than in untreated plants, indicating that BAP negatively affects the endoreduplication cycle (see multicellular and branched trichomes). Moreover, Maes et al. [131] proved that gene expression of *GL1*, *MYB23*, *GL3* and *EGL3* is also stimulated following BAP treatments. On the other hand, CKs also increase trichome formation during the reproductive stage; in fact, this class of hormones promotes trichome complement of the inflorescence stems [109].

Traw and Bergelson [132] first showed that mechanical wounding and JA significantly induce trichome development in plants. JA participates in trichome differentiation by degrading Jasmonate ZIM-domain (JAZ) proteins, as well as abolishing the interactions between JAZ with the bHLH and MYB factors, to promote the expression of trichome activators [9,133] (Figure 1). JA and SA enhance the resistance of plants to pathogens and pests attacks, and in *Arabidopsis*, they are also involved in the formation of the trichome complement [132,134]. JA has a positive effect on the density of trichomes on the leaf as well as on the accumulation of anthocyanins. However, mutants deficient in JA can differentiate trichomes [133]; therefore, JA appears not crucial for their development. It is likely that the influence of JA on trichome development could be specie-specific or linked to the trichome types.

In *Artemisia annua*, HOMEODOMAIN PROTEIN 1 (AaHD1), a HD-ZIP TF, which positively controls both glandular and non-glandular trichome initiation, was identified [135]. In particular, *AaHD1* knockdown lines showed a reduced sensitivity to JA on trichome initiation, which indicated that AaHD1 plays a key role in JA-mediated glandular trichome initiation [135]. Notably, in *A. annua*, artemisinin, the most potent medicine for malaria [136], is synthesized, stored, and secreted by trichomes. Tan et al. [137] showed that TRICHOME AND ARTEMISININ REGULATOR 1 (TAR1), an APETALA2 TF, play important roles in regulating both trichome development and artemisinin biosynthesis. *TAR1*, which encodes a protein mainly located in the nucleus, is predominantly transcribed in young leaves, flower buds, and trichomes. Notably, Tan et al. [137] also demonstrated that *AMORPHA-4, 11-DIENE SYNTHASE* (*ADS*) and *CYTOCHROME P450 MONOOXYGENASE* (*CYP71AV1*), two crucial genes in the biosynthesis pathway of artemisinin, were likely the direct targets of TAR1.

Maes and Goossens [108] collected evident data about the effect on trichome development of JA, CKs and GAs in *Arabidopsis*, concluding that all three phytohormones promoted hair initiation, but cause divergent effects on trichome maturation and other leaf parameters. Furthermore, they found that the ability of the three phytohormones to control trichome initiation is conserved across angiosperms lineage but that, within a specific plant species, different regulatory networks might be activated to direct the formation of the various trichome types.

SA has an opposite effect with respect to GAs, CKs and JA, reducing the density of trichomes in *Arabidopsis* leaves, also reducing the effects of JA [132]. However, the negative cross talk between the jasmonate- and salicylate-dependent defense pathways on trichome production has not been observed in other species [138]. The recessive *constitutive expresser of PR gene5* (*cpr5*) mutant of *Arabidopsis* was identified in a screen for constitutive expression of systemic acquired resistance (SAR) and its phenotype is severely dwarfed. *cpr5* has a higher content of SA and sugar-conjugated SA in comparison to wild type. Interestingly, the trichome complement of *cpr5* leaves showed a significant reduction [139]. In particular, *cpr5* plants displayed leaf trichomes of reduced size and decreased branching. Furthermore, trichomes on *cpr5* mutants had a reduced birefringence, suggesting a difference in cell wall structure between *cpr5* and wild type trichomes. In fact, Brininstool et al. [140] demonstrated that leaf cell walls of *cpr5* plants contained significantly less paracrystalline cellulose and had an altered wall carbohydrate composition. Effects of *cpr5* on trichome size and nuclear DNA content were epistatic to the effects of mutations in *try* or over-expression of *GL3*, indicating that these regulators of trichome development were dependent on *CPR5* function for their effects on trichome expansion and endoreduplication cycle [140].

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Notably, in some species, prickles (deterrent structures against herbivore and insects) are the extensions or modifications of glandular trichomes. The Trihelix Transcription factor GT2-like 1 (GTL1), a key regulator of ploidy-dependent trichome growth and drought tolerance, can positively regulates defense genes and inhibit factors that mediate growth and development. In this contest, it is interesting that GTL1 coordinates genes involved in SA metabolism, transport and response [141]. Recently data collected by a differential transcriptomic analysis of epidermis of *prickly* and *prickleless* mutants in *Solanum viarum* (an important medicinal plant) revealed that expression of several defense regulators like ethylene, SA, and PR-proteins were significantly down regulated in *prickleless* mutants [142].

Ethylene manifests its effects above all on the complexity of the trichome acting negatively on the branching process. In fact, mutants with low levels of ethylene developed only simple trichomes [143]. It was suggested that an ethylene receptor gene, *ETHYLENE RECEPTOR 2* (*ETR2*) could influence microtubule formation of the cell cytoskeleton by acting upstream of *CHROMATIN ASSEMBLY FACTOR1* (*CAF1*) and *TRY* and its function appears strictly dependent on *GL2* and *GL3* activity [144].

Trichome formation is also affected by brassinosteroids (BR); in fact, the *Arabidopsis brassinosteroid, light and sugar1* (*bls1*) mutant, impaired in BR response, develops fewer trichomes on both abaxial and adaxial surfaces of the leaf [144]. In addition, this mutant is characterized by a pleiotropic phenotype: short hypocotyl, expanded cotyledons, short roots, compact leaf rosette, reduced height, delayed bolting and hypersensitivity to metabolized sugars [144].

In order to understand how hormones are involved in the formation of tomato natural defenses against insects, Campos et al. [145] analyzed also the trichome complement using different mutants showing that ethylene, GA, and auxin mutants likely indirectly modified the trichome density, through effects on epidermal cell area. For example, a striking reduction in trichome density was observed for the ethylene-overproducer mutant *epinastic* (*epi*). Nevertheless, the reduction in trichome density might also be accounted for indirectly through the increase in individual epidermal cell surface area [132], thus decreasing the number of cells in a given area. However, BRs and JAs directly affected trichome density. In particular, the (*dumpy*) *dpy* mutant (BR-deficient) showed enhanced pubescence [146], while the opposite phenotype was observed for the *jasmonic acid insensitive1-1* (*jai1-1*) mutant [145,147].

4. Regulation of the cell cycle and the complexity of the trichomes

Many plants produce multicellular and/or branched trichomes, the formation process of which consists (like unicellular hairs), of three phases: initiation with determination of cell fate, endoreduplication, expansion and morphogenesis. Following the determination of the cell fate, the progenitor cells of the trichomes stop the mitotic cycle to move into the endoreduplication phase a cellular condition in which the duplication of the chromosomes in the phase S (DNA synthesis) of the interphase does not follow the entry into the mitotic cycle to form two daughter cells with normal DNA content. Therefore, the cell will be endoreduplicated. With the entry into mitosis of the endoreduplicate cells, polyploid cells could be generated [148].

The endoreduplication event is the basis of muticellular branching and expansion processes that underlie the trichome complexity. Cells destined to become multicellular trichomes initially elongate and then divide perpendicularly to the epidermal surface, in a context of continuous cell division [149]. Analogously, the number of ramifications depends on the cellular content in DNA: more ramifications are found if there is a high level of endoreduplication, while the complexity of the hairs decreases where the levels of endopolyploidy are reduced. It has been hypothesized that the control of cell cycle also plays an important role in the initial development of the trichomes [7,9,150].

In *Arabidopsis*, the trichomes show three branches originated by a phase that includes four cycles of endoreduplication. The trichome branching is coordinated by genes that have different regulatory roles in the controls of the number of endoreduplication cycles and therefore, the determination of the number of ramifications through the alteration of the DNA content (Figure 2).

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Among others, the genes *GL3*, *TRY*, *RBR1*, *CELL CYCLE SWITCH 52A2/FIZZY-RELATED1* (CCS52A2)/*FZR1*, *CCS52A1/FZR2*, *SIM*, *STICHEL* (*STI*), *KAKTUS* (*KAK*), *POLYCHOME//UV-INSENSITIVE4* (*PYM/UVI4*) and *RASTAFARI* (*RFI*) appear to play a key role in the cell cycle [9,82,151-153]. Therefore, *GL3* and *TRY* genes, in addition to possess an important function in the initiation of trichomes, participate in the regulation of branching [154]. The *Arabidopsis gl3* mutants produce trichomes with reduced ramifications due to fewer cycles of endoreduplication, compared to *try* mutants, which are characterized by additional endoreduplication cycles and a high number of ramifications [154]. Therefore, a direct relationship between the genes that regulate the development of trichomatous cells and the endoreduplication cycles could be delineated.

One key cell cycle regulatory pathway depends by the RBR protein and the E2F/DP TFs [155]. In the *Arabidopsis* genome, there is a single *RBR* gene and a complex family of E2F/DP proteins [156]. Three E2F (named a, b, and c) possess the typical domain organization, including one N-terminally located DNA-binding domain (DBD), DP heterodimerization, transactivation, and RBR-binding domains [157]. They heterodimerize with either of the two DP proteins (a and b) to form an active TF [158]. Kosugi and Ohashi [159] also showed that E2Fa/DPa heterodimers operate mainly as transcriptional activators and regulate cell proliferation and endoreduplication. Desvoyes et al. [151] demonstrated that RBR restricts cell division during early leaf development. Rapidly, after the proliferative stage, pavement cells of leaves retain their ability to proliferate but maintain their fate. By contrast, other epidermal cell types, e.g. trichomatous cells, do not change their proliferation state or fate specification [151]. At later stages, once the switch to the endoreduplication cycle program has occurred, RBR mainly restricted the progression through extra endoreduplication cycles. Therefore, it was demonstrated that RBR-mediated regulation of the endoreduplication cycle by a growth stage dependent in *Arabidopsis* leaf development [151]. In addition, reduced transcription of *RBR1* was correlated to a major number of endoreduplication cycles, resulting in trichomes with greater ramifications and phenomena of hyperplasia in young leaves [151].

Both *CCS52A1* and *CCS52A2* are key players that promotes the exit from the cell cycle and entry into the endocycle leading to endoreduplication. *CCS52A1* expression is negatively regulated by the GT2-LIKE1 trihelix TF [160], whereas the CK-activated ARABIDOPSIS RESPONSE REGULATOR2 activates its transcription [161]. In addition, *CCS52A2* expression appears to be specifically repressed by the E2F TF DP-E2F-Like1 (DEL1), which acts as a negative regulator in the initiation of the endoreduplication cycle [152,162]. FZR2 controls the induction of early rounds of endoreduplication while the remaining rounds may be mediated by FZR1 and FZR3. Most but not all endoreduplications in *Arabidopsis* are mediated by the expression of *FZR1* and *FZR2*. However, Larson-Rabin et al. [153] showed that lower activity of *FZR2* reduced both the number of endoreduplication cycles and trichome expansion. By contrast, an over-expression of *FZR2* was sufficient to allow extra cycles of endoreduplication in epidermal cell of leaf, roots and flowers leading to an alteration of the trichome size [153].

The progression of the cell cycle in the differentiation of leaf trichomes is governed by some cyclin-dependent kinases (CDKs), a family of protein-serine/threonine enzymes that whose activity is functional to the binding its activators or inhibitors as well INHIBITOR/INTERACTOR OF CYCLIN-DEPENDENT KINASES/KIP-RELATED PROTEINS (ICK/KRPs) [163]. The main activators of CDKs are several types of cyclins, a very large family of proteins present in many species. Several types of cyclins and CDKs play their roles at different stages of the cell cycle [164,165] (Figure 2). For instance, D-type cyclin-CYCLIN-DEPENDENT KINASE A (CYCD-CDKA) complexes function at the GAP1 (G1)/S and G2/Mitosis (M) transitions while CYCA/B-CDKA/B complexes function at the G2/M transition [3].

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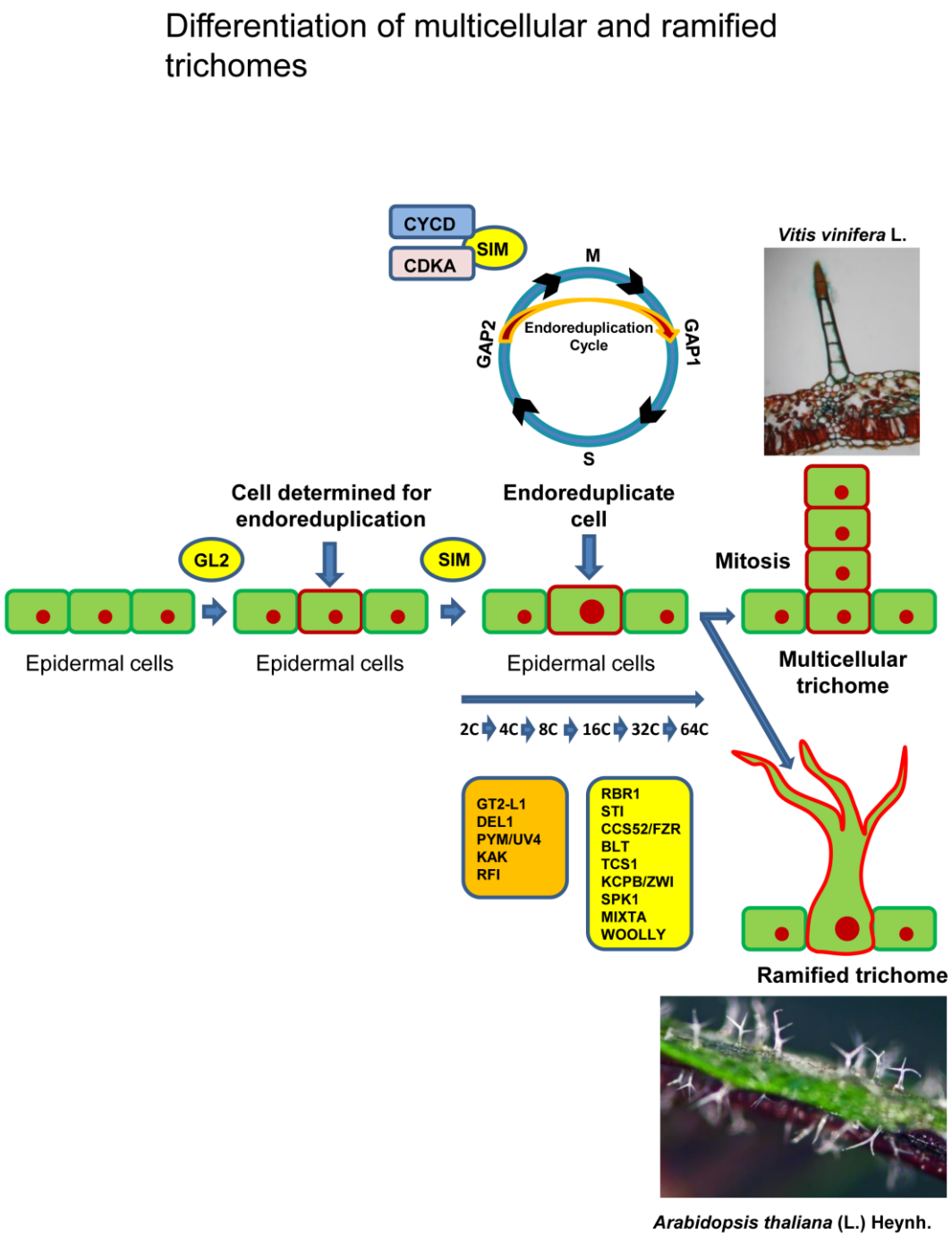

Figure 2. A simplified model in the differentiation of multicellular and ramified trichomes. An epidermal pavement cell activated by *GLABRA2* (*GL2*) become determined for trichome initiation (red cell wall). The *SIAMESE* (*SIM*) gene promotes the endoreduplication cycle (see also Figure 1). SIM interacts con D-type cyclin-CYCLIN-DEPENDENT KINASE A (CYCD-CDKA) complexes, which normally function at the G1/S and G2/M transitions, to repress the entry into the M phase. The nuclear DNA content increases from 2C until 64C. The endoreduplicate cell can follow two fates also in relation to the species: enter into the mitosis process originating a multicellular trichome as in the grape (*Vitis vinifera* L.) or originate branched trichomes as in *Arabidopsis thaliana* (L.) Heynh. In the orange box are indicate some negative regulators of

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the development of complex trichomes: GT2-LIKE1 trihelix (GT2-L1), DP-E2F-Like1 (DEL1), KAKTUS (KAK), POLYCHOME//UV-INSENSITIVE4 (PYM/UVI4), and RASTAFARI (RFI). By contrast, in the yellow box are indicate some positive regulators: RETINOBLASTOMA RELATED1 (RBR1), STICHEL (STI), CELL CYCLE SWITCH 52A2/FIZZY-RELATED1 (CCS52A2)/FZR1, CCS52A1/FZR2, BRACHLESS TRICHOME (BLT), TRICHOME CELL SHAPE 1 (TCS1), kinesin-like calmodulin-binding protein/ZWICHEL (KCBP/ZWI), BRACHLESS TRICHOME (BLT), SPIKE1 (SPK1), MIXTA and WOOLLY. S: DNA synthesis; M: mitosis.

Two genes fundamental to regulate the endoreduplication cycle are *SIM* and *STI* [166]. *SIM* encodes for a cyclin-dependent kinase inhibitor, that interacts with D-type cyclins (CYCDs) and CDKA to repress entry into the M phase (Figure 2), resulting in switch from mitotic to endoreduplication cycle [167]. Therefore, in *Arabidopsis sim* mutant plants, the trichomes are mostly multicellular but in reduced number per leaf [153,168,169]. The cyclins also contribute to the specification of the substrate of the cyclin-CDK complex, in fact, only specific "cyclin-CDK pools" promote the initiation of DNA replication, through the phosphorylation of the specific substrate for the transition, in this instance, from the G1/S or G2/M phases [170]. It was proposed that the expression of the *CYCLIN B1; 2* gene, which encodes a type B cyclin controlling the G2/M transition, was usually inhibited by the *SIM* gene [171]. In fact, the ectopic expression of *CYCLIN B1; 2* induces the formation of multicellular trichomes in *Arabidopsis* and in the *sim* mutant. The *CYCD3; 1* gene is specific for the formation of type D cyclin that in *Arabidopsis* trichomes induces cell division [172-173]. This gene is also directly inhibited by *SIM*. In addition, Schnittger et al. [171] have demonstrated that the *sim* mutant phenotype is rescued when *ICK1/KRP1*, a CDK inhibitor that interacts with CYCDs, is expressed in trichomes.

The regulation of the cell cycle during the development of unicellular trichomes differs from that relating to the formation of multicellular trichomes; however, in both cases the transition from mitosis to endoreduplication is fundamental. In *Arabidopsis*, mitosis is definitively inhibited and replaced by the subsequent endoreduplication phase, allowing the determination of cell fate and the development of unicellular trichomes. In tomato and other species characterized by multicellular hairs, mitosis is inhibited and similarly to the previous model, the endoreduplication phase is triggered; however, in the determination of cell fate, the pre-trichromatosis cells will resume some mitotic cycles, allowing the formation of multicellular trichomes [174].

By increasing the mitotic process, the development of multicellular trichomes is favored with respect to the initiation and activation of the trichomes themselves. It has been deduced that the inhibition of the initiation of the trichomes in *Arabidopsis* plants characterized by an increase in the mitotic cycle, was due to the inability of the activator complex to reach a threshold level sufficient to promote the determination of leaf follicles [175]. *STI* seems to play a key role on secondary branches. The *STI* gene encodes a protein containing a domain with a high similarity to the ATP-binding eubacterial DNA-polymerase III gamma-subunits [166]. In addition, the N terminal region of the product of *STI* also contain two PEST domains, and two nuclear localization signals (NLS) are placed at N terminal and C terminal region, respectively [176]. Xi et al. [176] also suggested that in *Arabidopsis* the PEST domain could be important for *STI* functioning in regulating trichome branching. This was deduced based on its direct interaction with the *BRACHLESS TRICHOME* (*BLT*) gene, an important linker of cell shape and endoreduplication cycle that interacts both genetically and physically with *STI* [176,177]. Although *blt* mutants have normal trichome DNA content, over-expression of *BLT* results in an additional round of endoreduplication [177]. In addition, loss-of-function mutations in *BLT* were found to enhance multicellular trichomes in *sim* mutants [177].

In *Arabidopsis*, Perazza et al. [178] have isolated five mutants, named *polychome* (*pym*), *rastafary* (*rfi*), *kaktus2* (*kak2*), *kak3* and *kak4* that shown, in comparison to wild type, leaf trichomes with an increased branching phenotype (five-six branches). These phenotypes were strongly reminiscent of both *try* and *spy* plants. An increased nuclear DNA content was detected in *pym*, *spy-5*, *kak*, and *rfi* trichomes giving new evidence for a link between endoreduplication and cell in trichome complexity [178]. *KAK*, *PYM* and *RFI* specifically repress the endoreduplication cycle in trichomes.

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Downes et al. [179] identified a family of seven HECT-containing ubiquitin-protein ligases (UPL1-UPL7). The mutants shown abnormal trichome morphology. Instead of developing three branches, many *upl3* trichomes contained five or more branches. The *upl3* trichomes also often undergo an additional round of endoreduplication resulting in enlarged nuclei with ploidy levels of up to 64C. Genetic analyses demonstrated that *upl3* mutants and *kak-2* were allelic. Therefore, Downes et al. [179] proved that the *KAK* gene represses the endoreduplication cycle, through the degradation of a specific protein, characterized by a ubiquitous system. The *KAK* gene recognizes a monophylogenetic subgroup of HECT proteins that also enclose Armadillo-like repeats [180].

PYM/UV-INSENSITIVE4 (*UVI4*) is a negative regulator of the anaphase-promoting complex/cyclosome (APC/C) ubiquitin ligase required for correct mitotic progression and cell fate determination, inhibiting premature cell differentiation [181]. Heyman et al. [182] showed that *uvi4* and *del1-1* mutants are characterized by an increased trichome branching phenotype. Notably, a quantification of the trichome nuclear size revealed an increase in the DNA content in *uvi4* mutant trichomes, similar to the *del1-1* mutant, and these evidences are indicative for a role of *DEL1* in suppressing endoreduplication in trichomes [182]. In the *uvi4;del1-1* double mutant, a clearly enhanced effect on trichome branching was observed, with a correspondent increased trichome nuclear size compared to the single mutant [182].

Several evidences suggest that the organization and dynamics of cortical microtubules (cMTs) are strictly linked with branching differentiation of trichomes [183-186]. cMTs displayed a high flexibility that depends on their ability to switch rapidly between states of elongation and shortening, which is controlled by microtubule-associated factors and the concentrations of assembly-competent α/β-tubulin heterodimers [187,188]. Mathur and Chua [184] proved that in trichome morphogenesis, the structure of cMTs drastically changed at the branching site. In addition, mutations in genes involved in the establishment of α/β-tubulin heterodimers or cMTs dynamics frequently lead to altered trichome branching [183,185,186,189-191]. Abe et al. [186] analyzed the semi-dominant *lefty1* and *lefty2* mutants of *Arabidopsis* originated from identical dominant-negative amino acid substitutions in α-tubulin 6 (TUA6) and α-tubulin 4 (TUA4). The *lefty* double mutant seedlings showed helical growth in hypocotyls and radial cell expansion in the root elongation zone with an abnormal organization of cMTs and a decreased trichome branching [186]. Abe and Hashimoto [190] showed that when a peptide sequence encoding the hemaglutinin (HA) epitope was attached to the N-terminus of TUA6 and expressed constitutively, transgenic *Arabidopsis* plants exhibited a semi-dwarf phenotype and low fertility. Notably, plants expressing HA-TUA6 protein showed cMTs more polymerization-prone and more branched trichomes [190].

TRICHOME CELL SHAPE 1 (*TCS1*) encodes a coiled-coil domain-containing protein that binds to microtubules and promotes the assembly of microtubules. TCS1 is physically associates with kinesin-like calmodulin-binding protein/ZWICHEL (KCBP/ZWI), a microtubule system involved in the regulation of trichome branch number [192]. Chen et al. [193] performed genetic analyses on *Arabidopsis* mutants demonstrating that *kcbp/zwi* was epistatic to *tcs1* with respect to trichome branch number. Therefore, it was suggested that TCS1 interacts with KCBP to regulate trichome cell shape by affecting the microtubule system stability [193].

Recently, Liang et al. [194] have identified an *Arabidopsis* mutant, *aberrantly branched trichome1-1* (*abt1-1*) characterized by a reduced trichome branching phenotype. *abt1-1* is a new allele of the *SPIKE1* (*SPK1*) gene, which encodes a member of the CDM family proteins that functions as a guanine nucleotide exchange factor (GEF). CDM is the acronym for the genes *Caenorhabditis elegans* CED-5, human *DOCK180*, and *Drosophila melanogaster* myoblast city [195,196]. Notably, Liang et al. [194] showed that *SPK1* was involved in the arrangement of nuclei in the trichome cells. In addition, it was identified the coordinated regulation of trichome branching by the interaction between *SPK1* and two other trichome branching regulators, *ANGUSTIFOLIA* (*AN*) and *ZWI* [194].

The *MIXTA* gene codifies for a TF R2R3-MYB that controls the determination of cells originating multicellular trichomes on petals of *Antirrhinum majus* [197]. Two homologues of *MIXTA*, *MYB MIXTA LIKE1* (*AmMYBML1*) and *CotMYBA* present in *A. majus* and cotton, respectively, promote the formation of multicellular trichomes also in *Nicotiana tabacum*, when

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ectopically expressed [9,37,197]. These results suggest that a *MIXTA-LIKE* gene can actively acts in the formation of multicellular trichomes in several different species [198,199]. Indeed, in view of that, these R2R3-MYB factors conserve the DNA binding domain like to GL1; it has been proposed that in some species the activity of *MIXTA-LIKE* genes replaces the role played by *GL1* in *Arabidopsis* [9]. In fact, Payne et al. [37] demonstrated that an over-expression of *GL1* on tobacco has no effect on the production of trichomes, such as the ectopic expression of *MIXTA* on *Arabidopsis gl1-1* mutants not generate trichomatous phenotypes. In fact, in *Arabidopsis*, the *MIXTA* genes (e.g. *NOK*) repress branching of trichomes [67].

In tobacco, Serna and Martin [200] hypothesized that MIXTA proteins do not require the interaction of *GL3* and *EGL3* to control trichome differentiation and therefore the MBW complex is not as fundamental as in *Arabidopsis*. Tomato is a very complex case, because this species differentiates eight morphologically distinct types of multicellular trichomes [14,201], out of which two types of non-glandular trichomes (III and V) and four glandular types (I, IV, VI and VII) that have been characterized [16]. Recently, Xu et al. [202] demonstrated that SlMYC1, a bHLH TF, was essential for type VI glandular trichome development. In addition, SlMYC1 differentially regulates mono- and sesquiterpene biosynthesis in the type VI glandular trichomes of tomato leaves and stems. Each type of trichomes follows different regulatory pathways; however, Yang et al. [203] demonstrated that the *WOOLLY* gene fundamentally controlled all types. As with the *MIXTA* gene, the over-expression of *WOOLLY* in *Arabidopsis* has no effect. These data demonstrate that multicellular trichomes of tobacco and tomato and unicellular trichomes of *Arabidopsis* and cotton are not homologous structures and different gene regulatory network [203] likely controls the pathways of development. It is assumed, therefore, that during evolution, genes such as *WOOLLY* and *PROTODERMAL FACTOR2* (*PDF2*) have acquired various biological functions among angiosperms [204]. *Arabidopsis* and cotton are Rosids while tobacco and snapdragon are Asterids. In the formation of trichomes, metabolic pathways, at least partly evolved differently at the time of their ancestral separation [201], probably control Rosids and Asterids.

It has not yet been elucidated whether and how far phytohormones can be involved in the development of multicellular trichomes; however, it seems that JA participates in their initiation, while CKs and GAs promote the outgrowth of multicellular hairs on tomatoes [204]. Moreover, it is now known that different types of trichomes present in the same species, are controlled by specific ways and different hormones; for example, the VI type of trichomes in tomato plants is specifically activated by JA, while the type VII by CK [108]. In tomato, also auxin seems to play a crucial role in the development of glandular trichomes. The *AUXIN RENSPONSE FACTOR* (*ARF*) genes encode a large family of proteins involved in auxin signaling transduction [205]. Zhang et al. [206] have showed that in tomato, *SlARF3* encodes a protein containing two conserved domains, B3 and ARF, and lacking an Aux/IAA domain. A down-regulation of *SlARF3* induced a decreased density of epidermal pavement cells and a reduced density of type I, V and VI trichomes of leaves, which suggested the important role of *SlARF3* in epidermal cell fate and in the formation of trichomes [206].

5. Epigenetic factors involved in trichome development

The accurate regulation of gene expression in space and time is fundamental for development of tissues, organs and whole organisms. The spatial and temporal expression profiles of many genes are controlled genetically by specific DNA sequences. Moreover, many aspects of development involve epigenetic regulation: mitotically and/or meiotically heritable yet reversible changes in gene expression without changes in DNA sequence. More than 130 genes encoding proteins involved in epigenetic regulation in plants have been identified [207]. These include: (i) regulator of DNA modification (i.e. DNA methyltrasferases, cytosine demethylation and DNA glycosylases, methylcytosine-binding proteins and proteins required for methyl group donor synthesis); (ii) histone-modifying enzymes and histone variants (i.e. histone deacetylases and histone acetyltransferases, histone methyltransferases and histone demethylases, histone variants, linker histones, and no histone proteins); (iii) polycomb proteins and interacting components; (iv)

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nucleosome-organizing proteins (i.e. chromatin-remodeling complexes and chromatin assembly factors); (v) small interfering RNAs (siRNAs)- and micro RNAs (miRNAs)-mediated post-transcriptional gene silencing (PTGS) [207,208].

The epigenetic state of the cell also appears to play a role in the final shape of the trichome based on loss-of-function mutations to the trimeric protein CHROMATIN ASSEMBLY FACTOR1 (CAF1) [209,210]. CAF-1, consisting of three subunits, p150, p60, and p48, has been originally purified from nuclei of human cells as a factor, which provided for the assembly of nucleosomes expressly onto replicating DNA *in vitro* [211]. Since CAF-1 is associated with newly synthesized histones H3.H4 and localized at replication foci in proliferating human cells, CAF-1 was thought to be involved in chromatin assembly during DNA replication and DNA repair *in vivo* [212-214]. Mutations either to the *FASCIATA1* (*FAS1*) or to the *FAS2* subunits of CAF1 showed increased trichome branching and are thought to act through regulation of *STI* [210]. However, characterization of the trichome morphology on rosette leaves of *fas2-1;kak-2* double mutant plants revealed that the two alleles were not epistatic. By contrast, Exner et al. [210] suggested that CAF-1 controls trichome branching independently from the *KAK*-containing pathway. In particular, it was supposed that CAF1 and *STI* controlled trichome differentiation in an endoreduplication-independent pathway [210]. In addition, while CAF-1 was not needed for the normal endoreduplication in WT trichomes, CAF-1 was required for the extra frequent series of endoreduplication cycles that occur in *kak* mutants. Loss of CAF-1 function also caused an increased transcription of the *H3.2* gene encoding for the histone H3-2. Exner et al. [210] proved that chromatin of *CAF-1* mutant trichomes contained increased amounts of the H3.2 variant histone. Moreover, because *CAF-1* mutants showed increased trichome branching but normal endoreduplication, it was likely that *CAF-1* restricted the ramification during trichome maturation independently from the endoreduplication cycle [209].

The state of chromatin packaging is controlled during growth and development and can regulated both temporal and spatial gene expression [215]. One form of control is through the covalent modification of histone proteins by the addition of acetyl groups, usually in the N-terminal domain of the histones [216]. Transcriptional coactivator complexes including a histone acetyltransferase (HAT) control histone acetylation. The well-characterized histone acetyltransferase GENERAL CONTROL NON-REPRESSED PROTEIN5 (GCN5) physically interacts with the transcriptional adaptor protein ADA2; the fundamental nature of their interaction was supposed by the lack of acetylation when ADA2 was absent [217]. A recent investigation demonstrated that GCN5 is also involved in the regulation of trichome initiation by modulating the transcription activities of trichome initiation regulator genes via H3K9/14 acetylation [218]. In fact, a mutation of the *GCN5* gene led to increased leaf trichome number in *Arabidopsis*. Expression analyses showed that *CPC*, *GL1*, *GL2*, and *GL3*, were down regulated in the *gcn5* mutants. In addition, ChIP assays indicated that these four trichome initiation regulator genes are direct targets of GCN5. In accordance with these data, Wang et al. [218] also demonstrated that GCN5-mediated H3K14/K9 acetylation levels on the Transcription Start Site (TSS) motifs of these genes were decreased.

In *Arabidopsis*, GCN5 and ADA2b are also required to connect endoreduplication and trichome branching [219]. ADA2b and GCN5 play specific roles in leaf tissue, affecting cell growth and division in rosette leaves often in complex and even reverse directions. Kotak et al. [219] demonstrated that *gcn5* mutant leaves displayed overall reduced ploidy levels, while *ada2b-1* mutant leaves showed increased ploidy. It was also proved that *gcn5* and *ada2b* mutants were characterized by alterations in the number and patterning of trichome branches, with *ada2b-1* and *gcn5-1* trichomes being significantly less branched with respect to normal plants, while *gcn5-6* trichomes showed increased branching [219]. Therefore, ADA2b and GCN5 were required to link nuclear DNA content with cell growth and morphogenesis of *Arabidopsis* leaves and trichomes [219].

5.1. miRNAs and trichome development

Micro RNAs (miRNAs) are small, endogenous, non-coding RNAs of 20-22 nucleotides in length and are present in plants, animals, and protozoa [220-222]. miRNAs modulate the expression of their target genes at the post-transcriptional level controlling many aspects of cellular functions

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[5,223,224]. miRNAs were also found to regulate the various TFs and genes involved in the trichome biogenesis [5,225]. Some explicative examples can be illustrated. In *Macuma pruriens*, the unicellular trichomes showed the flowing fluid or cytoplasm inside the trichome. Trichomes were found on various parts of the plants, but they were not uniformly distributed. Trichome density on the pod was the highest likely to protect the seeds from various insects that can harm such as pod borers or animals [226]. *M. pruriens* miRNAs (Mpr-miRNAs), which were found to regulate the genes and TFs governing trichome initiation and differentiation in this species, have been identified [226]. In particular, Singh et al. [226] proved that mpr-miRNA 1513 was involved in regulation of TRASPARENT TESTA 1 (TT1) TF while mpr-miRNA 2673 was found to regulate the GL3 and anthocyanin regulatory proteins. These miRNAs also showed pleiotropic effects, regulating other genes as well as *GL1*, such as *GL2* and *CPR-5* [226].

The medicinal plant *Xanthium strumarium* is covered with glandular trichomes, which are the sites for synthesizing pharmacologically active terpenoids such as xanthanolide, which possess antifungal, antibacterial, and cytotoxic activities, and exhibit a growth inhibitory activity against insects [227-229]. Based on the *X. strumarium* transcriptome data, Fan et al. [230] suggested that some of the differentially expressed *miRNAs*, including *miR6435*, *miR5021* and *miR1134*, might be involved in terpenoid biosynthesis in glandular trichomes.

The *SQUAMOSA* genes *PROMOTER BINDING PROTEIN LIKEs* (*SPLs*) have roles in leaf development, vegetative phase change, flower and fruit development, plant architecture, sporogenesis, GA signaling and toxin response [231]. In addition, *SPLs* promotes the expression of *TLC1* and *TRY* through a direct link with their promoters, who participate in a temporary control on the differentiation of trichomes [232]. Activity of several *SPL* genes are post-transcriptionally regulated by miR156, expression of which decreased in an age-dependent manner [233-236]. The subsequent increase in *SPL* activity contributes to a gradual reduction in trichome initiation on younger cauline leaves, stem internodes and sepals [232,237], as well as to trichome formation being shifted from the adaxial to abaxial side of leaves [26,112]. Effects of the miR156/*SPL* system on gene regulation were also discovered in other species, including *Oryza sativa* [238], *Brassica napus* [239], *Panicum virgatum* [240], *Medicago sativa* [241] and *Solanum tuberosum* ssp. *andigena* [242]. In *Arabidopsis*, genetic evidence indicated the involvement of *AtSPL9* in petal trichome initiation via activation of *TCL1* and anthocyanin pigment accumulation in vegetative stems [232,243]. *TCL1* gene was also significantly down regulated by miR156OE in alfalfa plants [244]. In *Nicotiana tabacum*, Zhang et al. [245] have identified three expressed sequence tags (ESTs) encoding miR156-targeted *SPLs* (*NtSPL2*, *NtSPL4* and *NtSPL9*). In *N. tabacum* plants over-expressing miR156, SEM analyses indicate that transgenic leaves produce a reduced number of trichomes in comparison to wild type in both early and late leaves [245]. These results revealed that over-expression of *miR156* causes plants exhibiting juvenile characteristics, thereby delayed juvenile-to-adult phase transition [245]. Therefore, the epidermal cell differentiation pattern could be used as a universal mark for juvenile-to-adult phase transition, although the morphological features of trichomes that distinguish these phases likely differs between species. In *Arabidopsis*, by dissecting the regulatory network controlling trichome formation on stem, Xue et al. [246] showed that a group of GRAS family TF members, LOST MERISTEMS 1 (LOM1), LOM2 and LOM3, targeted by timing miR171, operated in modulating the SPL activity through direct protein-protein interaction. Xue et al. [246] suggested that LOMs promote trichome formation through attenuating the SPL activity of trichome inhibition. In particular, it was demonstrated that LOMs shaping trichome distribution was dependent on SPLs, which positively regulate trichome repressor genes *TCL1* and *TRY*. In addition, Xue et al. [246] provided evidence that *MIR171* gene expression was regulated by its targeted LOMs, originating a homeostatic feedback loop.

The most widely cultivated cotton is an allotetraploid species (*Gossypium hirsutum*) that contains the homoeologous genes *GhMYB2A* and *GhMYB2D* that are functionally homologous to *Arabidopsis GL1* [247]. In cotton, Xie et al. [248] identified at least seven unique *miRNAs* and eleven *trans-acting siRNA* (*ta-siRNA*) candidate genes that participate in trichome regulatory interaction network. In addition, results collected from genomic, genetic, transgenic and mutant experiments

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suggested that functional divergence between *GhMYB2A* and *GhMYB2D* genes was mediated by miR828-directed ta-siRNA production, which regulated leaf trichome development in *Arabidopsis* and potentially cotton-fiber development [248].

The essential oil of mint (*Mentha* spp.) is stored in glandular trichome [11,249]. Singh et al. [225] demonstrated that several TFs including MYB families were regulated by miR5021. In addition, bHLH TFs were detected to be regulated by miR156 while myelocytomatosis viral oncogene homolog (*MYC*), a positive regulator of trichome initiation in *Arabidopsis* [86], was regulated by miR5015. Finally, WD rich proteins, involved in the cell fate determination, cell cycling and cell signaling were showed to be regulated by miR5015 [250]. Therefore, in mint, each component of the trimeric MBW activator complex was regulated by different miRNAs.

Reports are available for the mutation in the *SPY*, the repressor of GA signaling *locus*, which results in increased trichome formation [5,112,113]. Singh et al. [225] proved that in mint three miRNA families controlled the regulation of SPY: miR156, miR5015 and miR5015. Auxin responsive factor (ARF) is regulated by miR160 as reported in *Arabidopsis* and *Oryza sativa* [115,251]; whereas auxin induced protein, (IAA4) is regulated by miR414, which showed a response to stress. In mint, Singh et al. [225] proved that ethylene, with a role for trichome branching, was regulated by miR5021. Together, these observations suggested that in several species the temporal control of trichome development was regulated by the miRNA activities.

6. Conclusions

Trichomes are useful systems for studying cellular differentiation and development at the molecular level. The initiation of these highly specialized epidermal protrusions is spatially and temporally controlled; therefore, important molecules involved in heterochronic processes can be identified through the investigations on activation/repression of trichome initiation. Trichomes attracted first botanists and played interest in plant taxonomy especially, in the past. Actually, these structures are not only studied in plant ecology and plant protection but represents useful pharmaceutical factories and they will inspire in the future non-conventional human applications [252]. Control of developmental processes in trichome differentiation is a very complex issue where molecular players act in different regulatory networks: several TFs (both activators and/or repressors of initiation and cell cycle), hormones and epigenetic factors. The molecular basis of trichome development has been obtained especially in the *Arabidopsis* model but peculiar aspects of transcriptional regulatory network must be considered in other species. BAP, GAs and JA are major phytohormones with roles in trichome development; however, the mechanisms by which they are integrated with TFs remain largely unknown. Analogously, few details about the regulatory network that controls the development of glandular secretory trichomes of crops and medicinal plants are defined. As pointed by Pattanaik et al. [5], the genomic database TrichOME (www.planttrichome.org), can be useful source information to investigate the molecular origin of different trichomes types. Finally, the control of trichome development at post-transcriptional level and the epigenetic factors involved in this phenomenon are only partially known and future research will be required to obtain a wider perception of the trichome complement control.

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