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The modification of plant hormone level and signaling as a tool in plant biotechnology

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4

5 **Modification of plant hormone levels and signaling as a tool in plant biotechnology**
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12 **Fabiana Csukasi, Catharina Merchante and Victoriano Valpuesta**
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14
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16
17 *Departamento de Biología Molecular y Bioquímica, Universidad de Málaga, 29071*

18 *Málaga, Spain*
19
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21

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28
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34 Corresponding author:
35

36 Victoriano Valpuesta
37

38 Professor
39

40 Departamento de Biología Molecular y Bioquímica, Univesidad de Málaga,
41

42 29071 Málaga, Spain
43

44 Phone: 34 95 213 1932
45

46 Fax: 34 95 213 4267
47

48 e-mail: valpuesta@uma.es
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1. Abstract

Plant hormones are signal molecules, present in trace quantities that act as major regulators of plant growth and development. They are involved in a wide range of processes such as elongation, flowering, root formation and vascular differentiation. For many years, agriculturists have applied hormones to their crops to either increase the yield, or improve the quality of the commercial product. Nowadays, the knowledge of hormone biosynthesis, degradation and signaling pathways has allowed the utilization of biotechnological tools to improve even more the main agricultural crops. Natural or artificial mutants, with impaired functioning of the corresponding genes, have been adopted by their superior phenotype in specific agricultural traits. Also, transgenic plants have been generated to regulate internal hormone levels, or their signaling pathways, resulting in some crops that have revolutionized the agriculture.

2. Introduction

Plant hormones are signal molecules, present in trace quantities that act as major regulators of plant growth and development. Changes in concentration and cell/tissue sensitivity have dramatic effects in the physiology of the plant. This is a consequence of the fact that they participate in many different developmental processes that range from general growth, to flowering, root formation and vascular differentiation [1]. External application of these hormones has been for many years the procedure used in many agricultural crops to either increase the yield, or to improve the quality of the commercial product [2]. Nowadays, biochemical studies and genetic dissections have supported new insights into the molecular mechanisms of hormone biosynthesis and signal transduction pathways [3, 4]. This new information has allowed affording with new tools the improvement of main agricultural crops.

Known plant hormones are auxin, gibberellins, cytokinins, abscisic acid, ethylene, brassinosteroids, and more recently strigolactones [5, 6]. Not all of them have received the same attention as putative targets for plant biotechnology. Effectively, structural diversity of hormones is high, and so is the complexity of the metabolic pathways for their synthesis and catabolism, and not all of them are completely known [3]. The biggest aim of plant hormones Biotechnology has been to modulate their internal concentrations in a tissue/development specific manner. This is achieved after modification of either synthesis or degradation pathways, or both, since steady state level results from the net balance of these two processes. The second limitation has been determined by the same complexity of the signaling pathways for the different hormones. Whereas metabolic routes for hormone syntheses are diverse, their signaling pathways might overlap/interact [7]. This means that simple changes have frequently

1
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3 pleiotropic effects, which are difficult to explain at the whole organism level. Therefore,
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5 this review focuses on those cases where benefits are clear, as are also the fundament of
6
7 the biotechnological application.
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9

10 11 12 13 3. Gibberellins

14
15 Gibberellins (GAs) are tetracyclic diterpenoid compounds that control diverse
16
17 aspects of plant growth and development, such as seed germination, stem elongation,
18
19 leaf expansion and flower and fruit development. More than a hundred of different GAs
20
21 have been identified in plants and fungi but only a few of them are biologically active,
22
23 such as GA₁, GA₃, GA₄ and GA₇ [8, 9].
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27 GA biosynthesis occurs in three different compartments: plastids, endoplasmic
28
29 reticulum and cytosol. In first place, in plastids geranylgeranyl diphosphate (GGDP)
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31 acts as a precursor to *ent*-kaurene, then in the endoplasmic reticulum this *ent*-kaurene is
32
33 converted to GA₁₂ by two different cytochrome P450 monooxygenases, and finally
34
35 GA₁₂ is converted to GA₄ through oxidations on C-20 and C-3 by GA 20-oxidase
36
37 (GA20ox) and GA 3-oxidase (GA3ox) respectively in the cytosol [8, 9].
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39

40
41 Cereal crop yields have increased since 1960s because of the introduction of
42
43 high-yielding varieties of wheat and rice and the application of fertilizers and pesticides.
44
45 All these changes generated what was called the “Green Revolution” [10]. The problem
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47 with the application of fertilizers to traditional varieties became clear quickly for
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49 farmers; it did not increase their yield because plants grew too tall and fell over, process
50
51 known as lodging. What farmers needed were new varieties that gave increase grain
52
53 yield rather than increased plant biomass with the application of fertilizers [10].
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55

56
57 In rice, the dwarf genes originated from a Chinese cultivar, Dee-geo-woo-gen,
58
59 that was then used to produce the Taichung native 1 (TN-1) and later the IR-8 (“miracle
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3 rice”). TN-1 and IR-8 have been used also to produce some *indica* and *japonica*
4
5 cultivars. The semi-dwarf phenotype is the result of different alleles of a single
6
7 recessive gene, *sd1* [11]. The *sd1* mutation is recessive and normal height could be
8
9 restored by GA application, indicating that they were defective in GA production. In
10
11 2002, the *sd1* gene was identified as encoding a GA20ox. Dee-geo-woo-gen rice,
12
13 possessing the *sd1* allele, presents a 383-bp deletion in the *GA20ox* gene (*OsGA20ox2*)
14
15 that introduces a stop codon, producing a truncated enzyme [12]. As *GA20ox* are
16
17 members of a gene family and present functional redundancy, loss-of-function *GA20ox*
18
19 mutants present only a semi-dwarf phenotype compared with severely GA-deficient
20
21 plants that are completely dwarfed and sometimes sterile [11].
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27 The rice Tan-Ginbozu (*d35^{Tan-Ginbozu}*) is another example of increased yield by
28
29 semi-dwarf traits. The *d35^{Tan-Ginbozu}* was first believed to be blocked in the synthesis of
30
31 *ent*-kaurene or an earlier reaction. Years later, it was confirmed that the mutation in
32
33 *d35^{Tan-Ginbozu}* inhibited the activity of *ent*-kaurene oxidase (KO), that catalyzes the three
34
35 oxidative steps from *ent*-kaurene to *ent*-kaurenioc acid [13].
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39 The study of GA biosynthesis has extended to fruit plants. Some fruit trees can
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41 grow very tall if they do it on their own roots, so many of them are grown as scions
42
43 grafted on to a dwarfing rootstock. These trees are smaller and easier to handle, they
44
45 also present earlier flowering time and reduced vegetative growth. However, for many
46
47 crops, rootstocks are not enough and chemical growth retardants are used to prevent
48
49 excessive vegetative growth. In apple, inhibition of *MpGA20ox1* by sense or antisense
50
51 suppression resulted in dwarf plants with much lower levels of GA₁. The dwarf
52
53 phenotype was maintained when the transgenic scions were grafted on to invigorating
54
55 rootstocks. This technology could be very useful to improve the utilization of rootstocks
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3 to prevent plants to grow vigorous and to reduce the need for chemical growth retardant
4 application [14].
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8 In the citrus hybrid, Carrizo citrange, used as a rootstock, plants
9 overexpressing sense and antisense *CcGA20ox* gene produced shorter and taller
10 phenotypes respectively [15]. Plants also showed thorns, organs of citrus in juvenile
11 stages, with abnormal characteristics, they were much longer in sense and shorter in
12 antisense transgenic plants. Internodes cell length was not altered in plants, indicating
13 that in citrus GAs promote cell division rather than cell elongation. When transgenic
14 plants were grafted on wild type rootstocks, the phenotype disappeared, contrary to
15 what was described in apple. This suggests that GA produced in the roots can
16 compensate the deficit of GA in the antisense transgenic aerial part and opens the
17 possibility of using these plants as dwarfing rootstocks [15].
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31 Although the biosynthesis of GA is very well understood since many years
32 ago, the way that plants perceive the hormone and then transmit the signal to the whole
33 plant is still not completely clear. The GA receptor, GID1, was discovered in rice in
34 2005 [16]. OsGID1 was characterized as a soluble receptor and loss-of-function
35 mutations in the gene were shown to produce plants with a severe dwarf phenotype and
36 loss of GA responsiveness. It was also demonstrated that the OsGID1 protein was able
37 to bind bioactive GAs but not inactive GAs and that OsGID1 interacted with the rice
38 DELLA protein, SLR1. [17, 18].
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50 A few months after the discovery of rice GA receptor, *Arabidopsis GID1*
51 orthologs were identified. There are three *Arabidopsis GID1* genes, *AtGID1a*, *b* and *c*,
52 and all of them showed GA binding affinity to biologically active GAs. *Arabidopsis*
53 *GID1* receptors show partial redundancy, triple mutant plants show a very severe GA-
54 insensitive phenotype, demonstrating a more important role for GA signaling in
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3 *Arabidopsis* growth and development than previously described. As in rice, *Arabidopsis*
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GID1s interact with DELLA proteins in a GA-dependent manner [18, 19].

DELLA proteins are repressors of GA signaling and modulate all aspects of GA-induced growth and development in plants [20]. DELLAs belong to the GRAS family of transcriptional regulators, presenting a C-terminal GRAS domain. A unique N-terminal region is what gives name to this family of proteins and is composed of five amino acids, DELLA, a short spacer region and other six amino acids, TVHYNP [17, 20, 21]. This region is known to be essential to normal GA response [22]. DELLA proteins are rapidly degraded in response to GA treatment, and deletion or amino acid substitution in this conserved motif confers GA-insensitive phenotypes [20].

As with biosynthesis mutants, characterization of mutants that are impaired in GA signaling has been crucial to understand the role of GAs in plant development, as well as their targeting for biotechnological achievements. In general, all GA-deficient mutants show a similar phenotype, they are dark-green dwarf plants, defective in leaf expansion, stem elongation and seed germination. In *Arabidopsis*, a *rga* null mutation (*rga-24*) partially suppressed the phenotype of the GA biosynthetic mutant *gal-3*. The *gai* mutant was identified because it did not respond to GA application. In contrast to *rga-24*, *gai-t6* null mutation did not suppress the *gal-3* phenotype, but in combination with *rga-24* completely suppressed the plant defects [10, 23]. The triple mutant (*gal-3/rga-24/ gai-t6*) had a slender phenotype but mutant seeds were still non-germinating and mutant plants still showed GA deficient floral defects. This suggests that there must be other genes controlling developmental processes, and these genes are the *RGL*. A triple null mutant (*rga/rgl1/rgl2*) rescued the floral developmental defect and fertility of *gal-3* [24]. The *gai-1*, *rgaΔ-17* and *rgl1* mutants all lack 17 amino acids of the DELLA domain, and have a GA-insensitive dwarf phenotype. The deletion in the protein makes

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3 them constitutive repressors of the GA response; the proteins are not degraded in
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5 response to GA as in the wild type [10, 23].
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8 Several mutants with GA-insensitive phenotypes have been identified in other
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10 species, like *Rht1*, *Rht2* and *Rht3* in wheat and *d8* and *d9* in maize. Many dwarf wheat
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12 lines, as Norin 10, were introduced in the USA from Japan. Norin 10 contained the
13
14 *Reduced height* genes (*Rht*), responsible of the high-yielding semi dwarf wheat [10, 11,
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16 25]. Maize mutant alleles *d8* also showed these characteristics. In 1999, Peng *et. al* [25]
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18 demonstrated that *Arabidopsis GAI*, wheat *Rht-1* and maize *d8* were functional
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20 orthologs. They also demonstrated that the mutations of the N-terminal domain of these
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22 genes were responsible for the less sensitivity of the proteins to GA [25].
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27 Although all of the *DELLA* genes were first identified in *Arabidopsis* and
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29 cereals, the study of GA signaling genes in fruit plants is becoming a priority.
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31 Parthenocarpy, fruit development without seeds, is a much appreciated characteristic
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33 because it ensures high yields in difficult environments with low temperatures or
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35 absence of cross pollinators. It has been for many years a main goal in breeding
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37 programs of fruit crops. Hormones, particularly auxins and gibberellins, are known to
38
39 promote fruit development without fertilization. In tomato there is one gene encoding
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41 for a DELLA protein, named *SIDELLA*. Silencing of this gene by antisense technique
42
43 resulted in slender plants with elongated flower trusses. The fruits were smaller and
44
45 elongated compared with the wild type. The silencing of this gene also produced
46
47 pathenocarpic fruits, but this phenomenon was facultative because hand pollination
48
49 restored wild type fruit phenotype [26].
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55 In grapevine, a dwarf mutant derived from de L1 layer of the apical meristem,
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57 produced inflorescences in the shoot instead of the tendrils that are normally formed.
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59 The mutated gene was identified as an ortholog of wheat *Rht1* and *Arabidopsis GAI*.
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3 The formation of inflorescences in the mutant demonstrates that in grapevine, GAs
4 inhibit floral development [27] instead of promoting it as in *Arabidopsis* [28]. The
5
6 knowledge of the regulation of the flowering process can be useful to try to control fruit
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8 development and therefore to improve the production.
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13 The introduction of the *Arabidopsis gai* mutant gene in apple, resulted in a
14
15 clear reduction in stem length, internodes length and node number [29]. This is
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17 important since in modern apple production, dwarf trees are commonly used for
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19 achieving high production efficiency. However, transgenic plants showed reduced
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21 rooting ability. Production of new cultivars or rootstocks with dwarf characteristics has
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23 been an important objective for apple breeding.
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29 4. Auxin

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31 Since its discovery 70 years ago, the compound indole-3-acetic acid (IAA or
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33 auxin) has been the most studied plant hormone. It is known to be involved in almost
34
35 every aspect of plant growth and development. Depending on the tissue, auxin can
36
37 promote cell elongation or cell division. The exact mechanisms and sites of auxin
38
39 biosynthesis remain unclear, but there are two accepted biosynthesis pathways, one
40
41 dependent and one independent of tryptophan [30].
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46 The first problem to study auxin biosynthesis was the inability to find auxin
47
48 deficient mutants, since the mutants that presented high levels of free auxin were sterile
49
50 or had very heterogeneous phenotypes. In addition, it was not possible to determine if
51
52 the elevated levels of auxin were caused by an effect in auxin biosynthesis or
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54 conjugation. In 2001, Zhao, Y. *et. al.*, [31] identified the *Arabidopsis yucca* mutants,
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56 they presented a phenotype characteristic of elevated auxin levels, as long hypocotyls,
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58 short roots with long root hairs. They demonstrated that YUCCA is a member of the
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3 flavin monooxygenase (FMO)-like family that catalyzes a limiting step in *Arabidopsis*
4 tryptophan-dependent auxin biosynthesis. They also showed that the YUCCA family of
5 proteins is composed in *Arabidopsis* plants by ten members [31]. There is another auxin
6 biosynthesis mutant in *Arabidopsis*, the *superroot (sur1)* that presents high levels of
7 both free and conjugated auxin, resulting in auxin related phenotypes. The *SUR1* gene
8 encodes a protein with homology to the tyrosine aminotransferases and may be involved
9 in auxin Trp-dependent and independent biosynthesis pathways [30].

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20 In rice, seven *YUCCA*-like genes have been identified. In plants overexpressing
21 *OsYUCCA1* IAA levels were increased while plants with antisense expression of the
22 gene presented dwarfed shoots and short roots with long root hairs. The results provided
23 evidence for a role of *OsYUCCA* in auxin biosynthesis as in *Arabidopsis* [32]. As with
24 GA genes, the modification *OsYUCCA* genes in rice can generate the desired dwarf
25 phenotype.

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34 Auxin is known to play an important role in fruit development and ripening both
35 in climacteric and non-climacteric fruits, presenting a peak after ovary fertilization [33].
36 In strawberry and raspberry, the introduction of *DhfH9-iaaM* gene fusion, composed of
37 the regulatory region of *DhfH9* gene from snapdragon and the *iaaM* coding region from
38 *Pseudomonas syringae* pv *savastanoi*, that is known to promote auxin synthesis, confer
39 parthenocarpic fruit development to eggplant and tomato and increase fruit production
40 and quality, resulted in an increased number of fruits per plant and also a higher weight
41 and bigger size of the transgenic fruits. The increase in fruit yield reached 180% in
42 *Fragaria x ananassa*, the cultivated strawberry, and 140% in *Fragaria vesca*, the wild
43 strawberry, while the increase was of 100% in raspberry. The use of *DhfH9-iaaM* gene
44 must be considered to improve productivity in these two *Rosaceae* species [34].
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3 The introduction of the *DhfH9-iaaM* transgene in Thompson Seedless (low shoot
4 fruitfulness) and Silcora (medium shoot fruitfulness) cultivars of grape resulted in an
5 increased number of inflorescences per shoot in transgenic plants for both cultivars, in
6 Thompson Seedless the number was doubled compared to the wild type plants [35].
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12 As what happens with auxin biosynthesis, many aspects of auxin signaling are
13 poorly understood. The auxin receptor TIR1 has recently been identified [36, 37]. In
14 contrast, auxin regulated transcription is better understood since the past decade. Two
15 big families of transcriptional regulators are known to be involved in this process, the
16 Aux/IAA and ARF (auxin response factors) families. Auxin, after its binding to TIR1,
17 promotes the interaction between Aux/IAA proteins and the ubiquitin protein ligase
18 SCF^{TIR1}, stimulating the degradation of Aux/IAA proteins. Actually, the auxin receptor
19 TIR1 is part of the SCF^{TIR1} ubiquitin ligase complex and binds auxin directly. The
20 degradation of Aux/IAA proteins allows ARFs to bind to auxin regulated genes,
21 activating or repressing their transcription [30].
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36 In tomato, the *diageotropica (dgt)* mutant was identified as being defective in
37 some auxin-mediated responses. It was demonstrated that the mutant presented an
38 inhibition of the auxin induction of the only tomato Aux/IAA gene identified at that
39 moment, *LeIAA* [38]. Years later, ten other *Aux/IAA* genes were identified in tomato,
40 some of them also showed a reduced auxin stimulation of gene expression [39]. The
41 inhibition of *SIIAA9* produced plants with simple leaves instead of the compound leaves
42 of the wild type, and most importantly it induced fruit parthenocarpy. Despite their
43 parthenocarpic character, fruits presented normal size, skin color and flesh consistency
44 and they showed no change in ripening process. *SIIAA9* antisense lines were auxin
45 hypersensitive, presenting hypocotyls elongation, reduced apical dominance and
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3 increased leaf vascularization [40]. Later, it was demonstrated that a single-base
4
5 deletion mutation in *SlIAA9* gene caused the tomato mutant phenotype [41].
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8 In potato, auxins are known to play an important role in tuber development.
9
10 Endogenous auxin levels are high prior and during stolon swelling and after that they
11
12 start to decrease gradually. The first potato *Aux/IAA* gene identified, named *StIAA*, was
13
14 shown to be induced in potato tubers after fungal infection, but it was not clear if the
15
16 infection by itself caused the accumulation of *Aux/IAA* mRNAs or if it was the result of
17
18 other signals released by the fungus or the plant [42]. Another *Aux/IAA* gene was
19
20 identified in potato, *StIAA2*. Transgenic plants overexpressing *StIAA2* did not show any
21
22 visible phenotype; in contrast, transgenic plants inhibiting the gene expression presented
23
24 several dramatic defects. Plants were much taller, they contained two to three more
25
26 internodes, presented petiole hyponasty and extreme curvature of growing leaf
27
28 primordia in the shoot apex. These phenotypes are both common and unique to the ones
29
30 found in other plants, like *Arabidopsis* and tomato, and evidenced the importance of
31
32 studying the function of *Aux/IAA* genes individually, and in different plant species [43].
33
34 In addition, overlapping functions among different *Aux/IAA* family members must be
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36 considered when affording a biotechnological benefit of modifying a single gene.
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43 *ARF* genes are expressed in a wide range of tissues and organs. Screens in
44
45 *Arabidopsis* T-DNA insertion mutations have identified many *ARF* mutants, but most
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47 of them did not show a distinctive phenotype. That is not the case with *ARF* double
48
49 mutants that usually present a clear phenotype, as delayed flower maturation, reduced
50
51 fertility, reduced lateral root number, root cap defects and abnormal root gravitropism
52
53 [44]. A mutation in the coding region of *AtARF8* resulted in the production of
54
55 parthenocarpic fruits in *Arabidopsis* [45]. This was also found to be the case in tomato,
56
57 in which the expression of *Atarf8-4*, an aberrant form of the gene, in transgenic tomato
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3 (Monalbo) resulted in a significant increase in the number and also in the size of
4 parthenocarpic fruits [46]. Tomato transgenic plants inhibiting the expression of *DR12*
5 gene, a member of the ARF family, showed a pleiotropic phenotype, including dark-
6 green immature fruit, blotchy ripening, enhanced fruit firmness and increased hypocotyl
7 growth [47]. Some of these changes might be relevant for the improvement of this fruit,
8 but it will be needed to dissect the functions of other genes involved in order to avoid
9 the pleiotropic effects.
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22 **5. Ethylene**

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24 Ethylene is a gaseous hormone that regulates many aspects of plant growth and
25 development, including seed germination, root and stem elongation, root hair
26 development, root nodulation, flower senescence, abscission, sex determination, fruit
27 ripening, programmed cell death, responses to biotic and abiotic stresses, gravitropism,
28 and interaction between roots and rhizosphere [48, 49].
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36 Ethylene is produced in plants from *S*-adenosyl-methionine (SAM), which is first
37 converted in 1-aminocyclopropane-1-carboxylate (ACC) by the action of ACC synthase
38 (ACS), and then oxidized to ethylene by the ACC oxidase (ACO). Both ACC synthase
39 and ACC oxidase activities are encoded by multi-gene families, and the transcription of
40 the different genes is induced under different environmental and physiological
41 conditions [50, 51].
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50 Ethylene is perceived by a family of membrane-localized receptors that are
51 homologous to bacterial two-component histidine kinases. The number of isoforms as
52 well as the expression profiles varies within the species. For instance, *Arabidopsis*
53 harbors five ethylene receptors, ETHYLENE RECEPTOR1 (ETR1), ETR2,
54 ETHYLENE INSENSITIVE4 (EIN4), ETHYLENE RESPONSE SENSOR1 (ERS1),
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3 and ERS2; compared to the six of tomato, LeETR1, LeETR2, NEVER RIPE (NR),
4
5 LeETR4, LeETR5, and LeETR6; but ethylene-binding studies indicate similar ethylene
6
7 affinities for all *Arabidopsis* and tomato receptor proteins [52]. In *Arabidopsis*, ethylene
8
9 receptors play a negative role in the ethylene pathway [53]. It has been found that the
10
11 REVERSION-TO-ETHYLENE-SENSITIVITY1 (RTE1) protein genetically interacts
12
13 with ethylene receptors for the regulation of the ethylene response [54]. In the absence
14
15 of ethylene, the receptors are continuously stimulating CONSTITUTIVE TRIPLE
16
17 RESPONSE1, CTR1, which is a Ser/Thr kinase, and in this way, inhibits the rest of the
18
19 downstream components of the pathway. When ethylene binds to the receptors, the
20
21 MAPK cascade is released and results in the activation of EIN2, a positive key regulator
22
23 in the ethylene transduction pathway, which transmits the signal to the EIN3/EIL family
24
25 of transcription factors [53, 55]. At the end of the pathway are the ethylene inducible
26
27 genes which expression is under the control of the ethylene response transcription
28
29 factors (ERF). ERF-type transcription factors are specific to plants and belong to the
30
31 large AP2/ERF family. ERFs provide a control point for integration of multiple signals
32
33 and for diversification of ethylene responses [56].

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36 The role of ethylene in promoting fruit ripening and flower senescence provided a
37
38 commercial incentive for early, successful approaches to down-regulation of its
39
40 production. It is well described that the ripening of climacteric fruits is initiated by a
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42 peak of autocatalytic production of ethylene. It is assumed that control of ethylene
43
44 production at the late stages of ripening will delay the processes occurring at this stage,
45
46 and consequently will extend the shelf life of the fruit. Tomato has been the model plant
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48 for genetic manipulation, although similar strategies have been used in melon and apple.
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50 In terms of synthesis, genes encoding for the biosynthetic steps as ACC synthase and
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52 ACC oxidase have been silenced, by both antisense technology and RNAi (RNA
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3 interference). Antisense inhibition of *LeACS2*, which also down-regulated *LeACS4*,
4 reduced ripening-related synthesis of ethylene to 0.1% of control fruit. The antisense
5 fruit displayed an abnormal pattern of ripening such as reduced lycopene accumulation,
6 delayed softening and a much reduced climacteric peak [57]. This antisense strategy has
7 also been used in apple transformed with the antisense versions of the apple ACC
8 synthase and ACC oxidase with the aim of studying the role of ethylene in apple
9 ripening [58]. They observed very low levels of ethylene synthesis in three year old
10 apple trees grown in the field, and hence fruit that ripened more slowly. One side effect
11 of this strategy was a decrease in the concentration of enzymes responsible for the
12 formation of flavour esters, an effect that may adversely affect consumer acceptance.
13 RNAi strategy has also been used to silence ACO, these transgenic tomato plants
14 produced fruit that released only traces of ethylene and had a shelf life of more than 120
15 days [59]. In addition, to interfere with the endogenous synthesis of ethylene the
16 heterologous expression of ACC deaminase, cloned from soil bacteria, has been
17 employed. Effectively, the microbial enzyme ACC deaminase converts ACC to
18 ammonia and α -ketobutyrate, both of which are further metabolized. By inserting the
19 ACC deaminase gene into tomato plants under the control of the CaMV 35S promoter,
20 fruits with delayed ripening were readily produced [60]. No other significant phenotypic
21 differences were observed between plants with high levels of ACC deaminase activity
22 and nontransformed plants.
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50 Another strategy used to interfere with the ethylene action has been the use of the
51 mutated ethylene receptor from *Arabidopsis etr1-1*. This is a dominant mutation that
52 confers ethylene insensitivity when expressed in heterologous plant species. Thus,
53 overexpression of *etr1-1* has been used to produce transgenic plants with diminished
54 sensitivity to the hormone [61]. Seedling growth, flower senescence, flower abscission,
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3 and fruit ripening were all affected by the presence of the *etr1-1* transgene in both
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6 petunia and tomato plants. Regarding other receptors, NR was the first ethylene receptor
7
8 discovered in tomato. The *nr* tomato mutant shows global and total ethylene
9
10 insensitivity [62]. As NR and LeETR4 genes are up-regulated during ripening, they
11
12 have both been targeted for antisense experiments. Antisense repression of the mutant
13
14 NR gene in the *Nr* background produced fruit that ripened normally indicating that the
15
16 NR ethylene receptor is not required for ripening and confirming the receptor inhibition
17
18 model of ethylene signaling [63]. By opposite, it is becoming apparent that LeETR4
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20 plays an important role in ripening. Down regulation of LeETR4 produces an ethylene
21
22 hypersensitive phenotype that includes accelerated ripening [64], whereas in
23
24 *Arabidopsis* increased ethylene sensitivity is not observed until three receptors have
25
26 been knocked out [65]. The tomato dominant mutant *Green ripe (Gr)* is specifically
27
28 defective in fruit ethylene responses. It has been found that the mutation is the
29
30 consequence of a deletion in the 5' flanking region of the gene and causes ectopic
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32 expression of GR [66]. Interestingly, GR and RTE1 are members of the same protein
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34 family, and GR could achieve the appearance of tissue-specific function of the different
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36 ethylene receptors if it interacts with specific receptors [67].
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44 The process of flower senescence, with the consequence of flower wilting and
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46 decay, is triggered in many plant species by ethylene. Thus, commercial plants of
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48 carnation, begonia, and torenia have been produced with an antisense *ACC oxidase*
49
50 construct. In all the cases there was a dramatic decrease of endogenous ethylene
51
52 production and the benefit of extended vase life of the cut flowers [55]. Also, ethylene-
53
54 insensitive carnation flowers were produced after transformation with the
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56 *Arababidopsis etr1-1* gene. Transgenic carnation cut flowers had three times the vase
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3 life of non-transformed flowers and lasted up to 16 days, which is longer than flowers
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5 treated with either inhibitors of ethylene biosynthesis or ethylene antagonists [68, 69].
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8 It has been established that ethylene response factor (ERF) proteins play important
9
10 regulatory roles in plant response to abiotic and biotic stresses. Among abiotic stresses,
11
12 drought is the most devastating constraint to crop productivity. Tobacco plants
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14 overexpressing JERF3, a tomato ERF protein, showed an enhanced resistance to
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16 drought, salinity and freezing [70]. Enhanced tolerance to drought and high salinity was
17
18 also observed in rice plants overexpressing TERF1, another tomato ERF protein [71].
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20 Regarding biotic stress, another field of biotechnological application of the control of
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22 ethylene production is in the plant defense system. Ethylene involvement in the plant
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24 defense is complex, and commonly coordinated to other plant hormones. Thus, it is
25
26 known that ethylene production might be an early response of plants to pathogen attack,
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28 being associated to defense reaction, but it is also known that some pathogenic fungi
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30 and bacteria are capable of producing ethylene as a virulence factor. This contradictory
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32 role played by ethylene has resulted in quite variable effects of the diminution of
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34 ethylene production and sensitivity in the plant sensitivity to pathogens [72]. For
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36 example, tobacco plants transformed with the *etr1-1* mutant gene from *Arabidopsis* are
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38 insensitive to ethylene and develop symptoms of wilting and stem rot when grown in
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40 nonautoclaved soil [73]. Regarding the signaling cascade, the *Arabidopsis* mutant *ein2*,
41
42 impaired in ethylene signal transmission, exhibits increased susceptibility to the
43
44 necrotrophic fungus *B. cinerea* and the necrotrophic bacterium *Erwinia carotovora* but
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46 shows no alteration in susceptibility to the biotrophic Oomycete *H. parasitica* and the
47
48 biotrophic bacterium *P. syringae* pv *tomato*. Similar phenotypic effects, plants more
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50 resistant/susceptible to some pathogen species with no effect to other pathogen species,
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52 were obtained following other strategies, like the overexpression of some *Arabidopsis*,
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3 tobacco or tomato ERF genes [74]. In general, these results indicate that ethylene and
4 ERF-like transcription factors are involved in defense responses of various plant
5 species, but that their effect on disease resistance depends on the specific plant-
6 pathogen interaction. However, it appears as an area for future developments of genetic
7 strategy, transgenic or mutants, for improving plant response to pathogens.
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15 Success has been variable, mainly due to two facts. First is that usually endogenous
16 enzymes are encoded by gene families, being the transcription of the various members
17 organ-, tissue-, and developmental-dependent. This makes unpredictable the final result
18 of the genetic engineering strategy. Second, which is related to the first, is that abolition
19 of ethylene production in the whole plant frequently have deleterious effects. Best
20 solution to these shortcomings has been the use of specific promoters [69].
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31 **6. Cytokinins**

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34 Cytokinin (CK) was initially discovered as the hormone that promotes cell
35 division; it is now known to influence many aspects of plant growth and development
36 [75]. Cytokinins (CKs) are a structurally diverse group of biological molecules whose
37 chemistry has been extensively studied. However, some aspects of their metabolism and
38 signaling pathways are still undefined. The natural cytokinins are adenine derivatives
39 that can be classified by the configuration of their N6-side chain as isoprenoid or
40 aromatic cytokinins [75]. Biotechnological applications in relation to this hormone have
41 rested on the possibility of changing their endogenous level in a tissue/organ specific
42 manner. Local levels of CKs are controlled by synthesis, transport, inactivation, and
43 oxidation. Among these, synthesis and oxidation have been the targets for
44 biotechnological plant improvement.
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3 In the synthesis pathway there is an ATP/ADP isopentenyltransferase (IPT) gene
4 that encodes for a rate-limiting step. In *Arabidopsis* seven *IPT* genes have been
5 identified, being the promoters of four of them suppressed by CKs [76]. The only IPT-
6 encoding gene known in *Petunia hybrida* [77] has been used to modify the internal level
7 of CKs. This has been performed in potato where CKs are known to influence tuber
8 initiation, growth and dormancy. Potato plants cultured *in vitro* showed a significantly
9 increased tuber biomass after cytokinin application [78]. The *Sho* gene, from *Petunia*
10 *hybrida*, encodes an enzyme for cytokinin synthesis [79]. Moderate expression of the
11 *Sho* gene in potato produced and enhanced shoot production, delayed tuber formation
12 with significant reduction in tuber size, and inhibition of tuber dormancy [79]. In
13 general, stimulation of shoot and stolon development can be beneficial, jointly with the
14 reduction of dormancy, for enhanced tuber production. However, as occurs with other
15 transgenic plants, there are deleterious effects derived from ectopic expression of the
16 transgene. This must be eventually overcome with the use of tissue/stage-specific
17 promoters, as above indicated.

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20 In the catabolic pathway there is a step catalyzed by a cytokinin
21 oxidase/dehydrogenase enzyme (CKX) [75]. This enzyme irreversibly degrades CKs by
22 cleavage of the unsaturated N6-isoprenoid side chain. This step plays a main role in
23 controlling CK levels in plants. Selection of the corresponding gene as a target for
24 genetic engineering was based on previous studies aimed to characterize in many crops,
25 including rice, quantitative trait loci (QTLs) responsible for grain number, and
26 eventually for higher yield. Grain number is an important trait that contributes directly
27 to crop yield. A QTL that increases grain number in rice, *Gn1a*, was identified. High
28 resolution mapping of this *Gn1a* identified as a possible candidate gene the one with a
29 predicted open reading frame with high similarity to cytokinin oxidase/dehydrogenase
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(*OsCKX2*) [80]. This was further confirmed by producing transgenic rice plants expressing different levels of *OsCKX2* and examining the grain yield. Lines with two copies of the sense strand of *OsCKX2* showed reduced grain number compared to the control, whereas lines with antisense strands, which had reduced levels of expression, showed higher grain number [80]. Grain number jointly with plant height is an important trait that directly contributes to grain productivity. Thus, the manipulation of *OsCKX2* in rice is relevant since food shortage still remains as one of the most serious problems in this century. It is envisaged that crop grain production will need to increase by 50% by 2025 to meet the expanding food demands of the growing world population [81].

7. Brassinosteroids

Brassinosteroids (BRs) constitute another class of plant hormones that participate in multiple plant physiological processes like cell division, cell elongation, vascular differentiation, root growth inhibition, and ethylene synthesis. Currently 42 brassinosteroid metabolites and conjugates are known [82], among them the most physiologically active is Brassinolide (BL). Metabolic pathways for BRs biosynthesis might vary depending upon the plant species, the developmental stage, and the BR structure. Two categories of changes have been reported in BRs metabolism: 1) structural changes of the steroidal skeleton; and 2) structural changes in the side chain. The most common modification in the side chain include hydroxylation and side chain cleavage. Less common modifications include dehydrogenation, glycosylation and demethylation [82]. The synthesis of the steroidal skeleton starts from an initial precursor, campesterol, and de BR intermediates undergo a series of hydroxylations, reductions and epimerization, and a oxidation leading to the brassinolide (BL), the most

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3 biologically active. The signaling pathway is being characterized. The receptor BRI1
4 was identified by positional cloning in a mutant of *Arabidopsis* (*bri1*) insensitive to
5 brassinosteroids [83].
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10 The importance of brassinosteroids in cell elongation has generated interest in
11 manipulating their levels. It was reported that exogenous application of brassinosteroids
12 to a number of vegetables increased yields [84]. Recently, main interest has been in rice
13 where they are known to influence both plant height and leaf erectness [85]. Erect
14 leaves enhance light capture for photosynthesis, what allows more dense plantings and
15 higher nitrogen storage. The result is an increase in yield.
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24 In the screening of a mutant rice population, generated by γ -ray irradiation,
25 retrotransposon-mediated, or chemical mutagenesis, a mutant was isolated, *osdwarf4-1*,
26 with erect leaves but without abnormal leaf, flower or grain morphology. The gene was
27 finally identified as *OsDWARF4* after characterizing the site of insertion of the
28 retrotransposon [86]. This gene is homologue of the *DWARF4* gene from *Arabidopsis*
29 that encodes a cytochrome P450 that catalyzes the C-22 hydroxylation rate limiting step
30 of brassinolide biosynthesis [87].
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41 Most plant genes for brassinosteroid biosynthesis exist as single copies, however
42 in rice there are two genes encoding the P450 C-22 hydroxylase, *OsDWARF4L1* that
43 acts during shoot elongation and reproductive development, and the *OsDWARF4* gene
44 that controls leaf inclination. Thus the *osdwarf4-1* mutant displayed increased erectness
45 of the leaves, without the severe phenotype of the double mutant *osdwarf4-1*
46 *l/osdwarf4l1* plants. Small field trials involving the wild type and the *osdwarf4-1*
47 mutant revealed that yield was higher for mutants, being the difference higher with the
48 increase of density of planting and the amount of nitrogen fertilizer [86]. Biomass
49 production of the *osdwarf4-1* plants was increased nearly 40% as compared with the
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3 wild type plants. Under normal or dense planting conditions the erect leaves of the
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5 mutants increased photosynthetic rates and as result the plants were more productive.
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8 Table 1 shows some prominent examples of biotechnological applications
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10 derived from changes either in the hormone levels or the signaling pathways.
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13 14 15 **8. Future prospects**

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17 It is accepted that rational approach to improve crop quality depends on a better
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19 understanding of plant biology, in this case of hormone biosynthesis and signaling.
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21 Genomic projects have deciphered the sequences of tens of thousands of genes in plant
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23 genomes, but it is needed to know which genes control the quality traits of the crops by
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25 their involvement in hormone action. The functional genomics tools are being used for
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27 this task that will pave the way for biotechnological applications. Among these, those
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29 related to reverse genetics are becoming more important. We consider here the main
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31 advantages and disadvantages of two of them: the preparation of transgenic plants and
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33 the generation of chemical mutants.
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39 Field growth of transgenic crops for commercial production has been
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41 continuously increasing to reach values over 120 M of hectares by 2007
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43 (www.isaaa.org/resources/publications). However, most of them are restricted to few
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45 species (soybean, maize, cotton, and canola) and few traits (herbicide and insect
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47 resistance). In terms of hormone balance one of the most conspicuous events was
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49 performed in rice, where tissue-specific expression of the *GA2ox* gene caused a dwarf
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51 phenotype without affecting grain yield. Ectopic expression of the transgene, with the
52
53 consequent pleiotropic effect, was avoided by using the promoter of another rice
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55 biosynthetic gene (*OsGA3ox1*) [88]. The need of tissue/developmental-specific
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57 promoters is a great challenge for the future development of transgenic plants that
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3 becomes even more important when hormone actions are targeted. Limitations to have
4 access to these promoters are: 1) lack of characterization of 5' regulatory regions; 2)
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6 lack of knowledge on the rules for promoter engineering; 3) unavailability of efficient
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8 systems for gene targeting/homologous recombination in plants [89].
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13 Future developments of commercially attractive transgenic plants would be
14 probably restricted to main crops, like cereals and some legumes, improved in traits
15 with benefits for developing countries. Rejection by most European countries of the
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17 technology of genetic modification, known as GMO, appears as a great shortcoming for
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19 future development of new transgenic plants, especially in quality traits appreciated by
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21 the consumers of the developed countries.
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27 Most transgenic plants commonly present a complete loss of function, or
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29 constitutive gain of function, phenotypes that make them unacceptable as commercial
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31 crops [11]. However, it should be reminded that there were two alleles in two species
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33 that were critical to bring about the Green Revolution [10]. This indicates that the
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35 alleles that cause just a moderate variation in the physiology of the plant are the most
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37 important for the development of new commercial varieties. Since natural variation for
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39 new alleles is limited, once again chemical mutagenesis is being used to look for new
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41 improvements in plant performance. The use of the technical approach known as
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43 targeted induced local lesions in genomes (TILLING) [90] will facilitate the job. Using
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45 this technique, point mutations of selected genes can be produced and screened for their
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47 phenotypic effect. There are examples where allelic variants are produced and screened
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49 by TILLING to generate the desired phenotype [91]. The possibility of modifying
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51 multiple alleles with non GMO technology will become more realistic in the future, as
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53 the genome sequencing projects and the refinement of what is known as system biology
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55 will increase the predictive value of specific genetic changes.
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For Peer Review

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Table 1.

Gene/Locus	Hormone involved	Species	Mutant/transgenic	Phenotype	Reference
<i>sd1</i> (<i>GA20ox</i> gene)	GA	rice	mutant	Semi-dwarfed plants	[12]
<i>Rht</i> (<i>DELLA</i> gene)	GA	wheat	mutant	Decreased plant height, response to GA and increased GA levels	[25]
<i>DhFH9-iaaM</i>	auxin	strawberry, raspberry	transgenic (sense)	Increased number of fruits per plant, increased fruit weight and size	[34]
<i>SHAA9</i>	auxin	tomato	transgenic (antisense)	Parthenocarpic fruits	[40]
<i>LeACS2</i>	ET	tomato	transgenic (antisense)	Reduced ripening-related synthesis of ethylene to 0.1% of control fruit, abnormal pattern of ripening	[57]
<i>LeETR4</i>	ET	tomato	transgenic (antisense)	ET hypersensitive phenotype including accelerated ripening	[64]
<i>etr1.1</i>	ET	carnation	transgenic (sense)	Cut flowers had three times the vase life of non-transformed	[68]
<i>Sho</i>	CK	potato	transgenic (sense)	Increased shoot production, delayed tuber formation, decreased tuber size and	[77]

				inhibition of tuber dormancy	
<i>osdwarf4-1</i>	BR	rice	mutant	Increased biomass and grain production	[86]

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Victoriano Valpuesta is Professor at the Department of Biochemistry and Molecular Biology of the University of Málaga. He received a PhD in Biochemistry from the University of Sevilla and he gained a postdoc position in Michigan State University, working with Professor Martin J. Bukovac. In 1982 he was granted with a position as Professor in the University of Málaga, where he achieved the tenure as full Professor in 1990. Main research interest during this period was enzymology of the amino acids metabolism in plants. Afterwards he has spent two sabbaticals in the University of California in the laboratory of Dr. Michael S. Reid , and the University of Queensland in the laboratory of Dr. José R. Botella. His current research goals are centered on molecular studies of developmental processes in plants, mostly in fruits. His most recent research addresses the use of functional genomics tools in the ongoing research on plant development.

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