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Review

Recent Progress in the Regeneration and Genetic Transformation System of Cucumber

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Abstract: Cucumber (*Cucumis sativus* L.), belonging to the gourd family (Cucurbitaceae), is one of the major vegetable crops in China. Conventional genetic breeding methods are ineffective for improving the tolerance of cucumber to various environmental stresses, diseases, and pests in the short term, but bio-engineering technologies can be applied to cucumber breeding to produce new cultivars with high yield and quality. Regeneration and genetic transformation systems are key technologies in modern cucumber breeding. Compared with regeneration systems, genetic transformation systems are not yet fully effective, and the low efficiency of genetic transformation is a bottleneck in cucumber cultivation. Here, we systematically review the key factors influencing the regeneration and genetic transformation of cucumber plants, including the selection of genotype, source of explants and forms of exogenous hormones added to the medium, the methods of transgene introduction and co-cultivation, and selection methods. In addition, we also focus on recent advances in the study of molecular mechanisms underlying important agronomic traits using genetic transformation technology, such as fruit length, fruit warts, and floral development. This review provides reference information for future research on improvements in cucumber varieties.

Keywords: cucumber; genetic transformation; *Agrobacterium tumefaciens*; transgenic plants; positive selection system; biotechnology; plant regeneration

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1. Introduction

Cucumber (*Cucumis sativus* L.) is a major vegetable crop with important economic value that is cultivated worldwide. It produces fruits rich with nutrients including various proteins, minerals, soluble sugars, and vitamins. The polysaccharides and flavonoids in the fruit play important roles in antioxidant activity, cancer prevention, and inhibition of liver inflammation [1,2]. In the past few decades, traditional genetic breeding techniques (crossbreeding and mutation breeding) have played an important role in developing new cucumber varieties with high yield and quality [2]. However, cucumber plants have a narrow genetic base and a low rate of genetic variation, making it difficult to obtain stable genetic characters or genotypes [3]. With the rapid development of molecular biotechnologies, some innovative molecular approaches and bio-engineering technologies have been applied to cucumber cultivation [2]. In addition, the completion of the cucumber genome sequencing work has greatly promoted research in cucumber genomics [2]. Using molecular markers, genome-wide association analysis, and transgenic and gene editing approaches, a number of genes related to leaf shape, leaf color, branches, fruit quality, yield,

disease, and stress resistance have been mined successfully, and some progress has been made in the molecular mechanism analysis of cucumber plant sex and flower development, fruit development, and shoot branching [2].

Genomic transformation is an important aspect of genetic engineering technologies. It is defined as an array of events including the selection of a desirable gene, delivery, integration into the plant cells, and expression, giving rise to a whole plant and final molecular identification (Figure 1). Among the variety of available DNA delivery systems, *Agrobacterium tumefaciens*-mediated transformation has been used primarily for optimization and trait improvement in cucumbers [4]. *A. tumefaciens* achieves genetic improvement by transferring a plasmid fragment containing the gene of interest (called T-DNA) into host cells, and transgenic plants are then regenerated by cell and tissue culture techniques [5]. The variety cv. Poinsett 76 is used more successfully than most [1]. For example, transformation efficiencies have been up to 21% using cotyledons of cv. Poinsett 76 mediated by *Agrobacterium* strain EHA105 [6]. The Xintai mici strain is also popularly used. Higher transformation rates have been achieved using its cotyledons and cotyledonary nodes mediated by *Agrobacterium* strain GV3101 [7,8]. Leaf and hypocotyl tissues can also be transformed successfully when matched to the appropriate genotype [1,9].

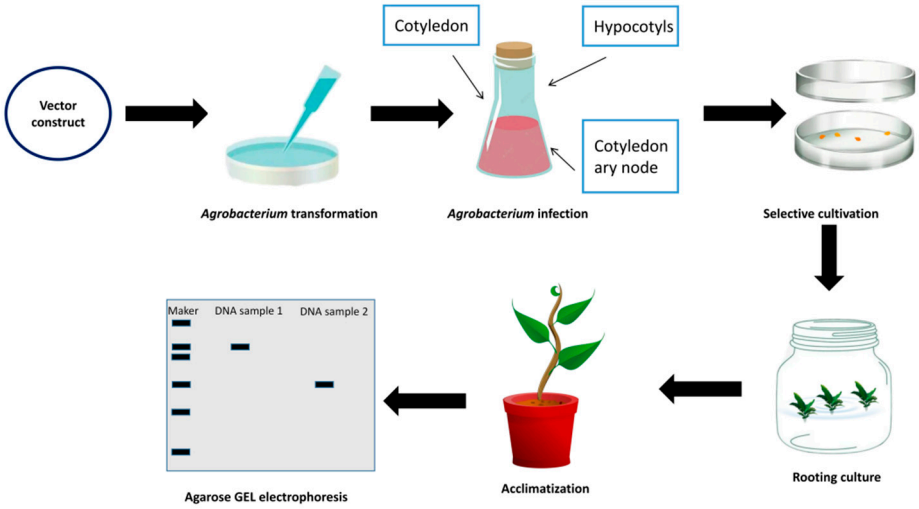

Figure 1. Schematic overview of *Agrobacterium*-mediated genetic transformation.

Although successful transformation was achieved decades ago in cucumber using *Agrobacterium*, progress in cucumber still lags [1]. High genotype dependence, requirement for exogenous hormones, selection of strain, infection method, and culture conditions before and after inoculation with *Agrobacterium* are the major constraints for successful regeneration and transformation. Here, we review recent progress on the influencing factors of cucumber regeneration ability and genetic transformation conditions and summarize the achievements of transformational approaches in the study of molecular mechanisms underlying important agronomic traits.

2. Factors Influencing the Regeneration of Cucumber Plants

Plant tissue culture is a potential tool for rapid and massive growth of plants (Figure 2) and has many advantages, such as not being disturbed by environmental factors, and little space is required to multiply large numbers of plants [10]. The regeneration of cucumber has been reported via organogenesis using different explants such as cotyledons [11], embryonal axis [12], nodal regions and shoot tips [13], and hypocotyls [14]. Indirect organogenesis procedures have also been reported using leaves, stems, and cotyledons [15]. According to an earlier report, the regeneration rate was largely dependent on the genotype of the cucumber strain, the nature of explants, and types of exogenous hormones used [10]. For breeding purposes, it is necessary to establish an efficient regeneration system.

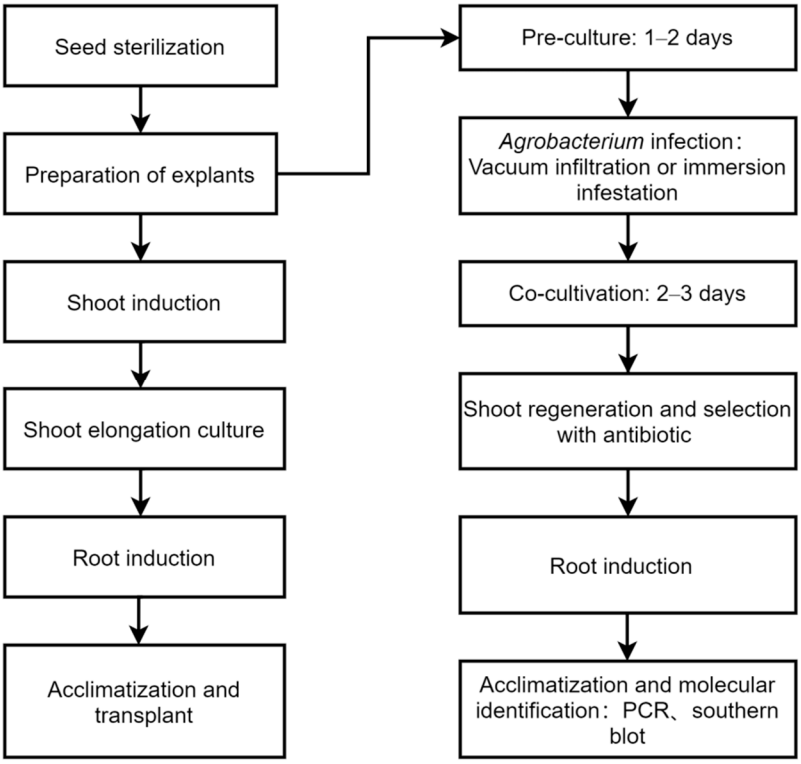

Figure 2. The protocol of in vitro regeneration (**left**) and *Agrobacterium*-mediated transformation (**right**).

2.1. Genotype of Explants

The genotype is an important factor affecting cucumber regeneration. The cucumber's cotyledon nodes from different genotypes cultured in Murashige–Skoog (MS) medium showed different differentiation ability [16]. In a study by Fan et al. [17], using medium supplemented with the same hormones, the regeneration frequency (proportion of explants that produced buds) of 'Nongcheng 3' could reach 100%, while that of 'Xinong 58' was only 58.3%. Some varieties cannot regenerate under most hormone combinations, while '9930′ can, and the bud induction rate (proportion of explants that produced shoots) was high: up to 96.7% [14,18]. Wang et al. [19] located the gene *Csa1G642540* closely related to cucumber regeneration in vitro. Quantitative real-time polymerase chain reaction (qPCR) analyses revealed that *Csa1G642540* was significantly more highly expressed in '9930′ (high cotyledon regeneration rate) than in '9930Gt' (low regeneration rate). For the first time, it was confirmed that the regeneration ability of cucumber is related to genotype [20]. Given the severe dependence on cucumber genotype, several varieties with high regeneration rates are often used in cucumber regeneration and transformation, such as '9930', 'Poinsett76', and 'Xintai mici' [21].

2.2. Explant Sources and Seedling Stages

Explants are in vitro culture materials cut from organs or tissues during tissue culture. Cotyledons, cotyledon nodes, hypocotyls, root, internodes, stem nodes, leaves, and protoplasts of cucumber have been used as explants to regenerate plants in vitro [1]. Liu [22] demonstrated that using cotyledon nodes as explants can lead to a higher induction rate of adventitious buds compared with true leaves, radicles, and hypocotyls. Zheng [23] changed the explants from stem nodes to cotyledons, and the bud induction rate increased from 63.5% to 78.3%. A higher frequency of shoot differentiation was also observed by Li [24], and shoot induction rate was as high as 96.2% when cotyledon nodes were used as explants. Thus, although hypocotyls and leaves can be used as explants for cucumber regeneration, the cotyledons and cotyledon nodes can often obtain a higher regeneration frequency and are used most frequently [25–27]. The elongation period of a typical cotyle-

don node is short; stem elongation and rooting are easy, but it is sensitive to antibiotics, so higher concentrations of antibiotics are often not added during screening so that the false positive rate is high. In contrast, cotyledons are induced to differentiate earlier and have a strong ability to divide. Such transformed cells are also more resistant to antibiotics, so it is advantageous to add higher concentrations of antibiotics to eliminate false-positive seedlings [28]. When cutting explants, it is necessary to pay attention to the seedling ages; given the different degrees of differentiation of explant cells at different developmental stages, the transformation efficiency is often affected [29]. It is generally believed that young material has a low degree of differentiation and is conducive to the induction of organogenesis. In related reports, 1–7 d was often used as the optimal seedling age. However, there is always a certain difference in the germination rate and potential of cucumber seeds, and the experimental materials and culture conditions in different studies are also different, resulting in different seed growth rates. Therefore, the seedling stage is used instead of seedling age to judge the inoculation period [22,30]. In many studies, the stage between cotyledon cohesion (two cotyledons detached from the seed shell) to two cotyledons flattened was selected as the best seedling stage according to the frequency of regeneration and the number of buds per explant [29–31]. In addition, Kim et al. [32] speculated that the explant size might affect the level of endogenous hormones in explants, which in turn affects the induction of adventitious buds. Rajagopalan and Perl-Treves [33] demonstrated that appropriate methods for cutting and inoculating explants facilitate the exposure of some potential meristematic cells to *Agrobacterium*.

2.3. Exogenous Hormone

During plant regeneration, the content of endogenous hormones is in a state of dynamic change. Therefore, callus differentiation and bud regeneration are greatly affected by the content and proportion of key hormones such as auxin and cytokinin (CTK) [34–36]. In bitter melon, relatively high zeatin (ZT) concentrations as well as low indole-3-acetic acid (IAA)/ZT appeared to be associated with bud formation [34]. A similar phenomenon was observed in barley, with the highest levels of IAA, trans-ZT, and cis-ZT among the varieties with the highest rates of regeneration [36]. The content of endogenous hormones in cucumber plants cultured in vitro was measured by Zhang [31], who observed that 0–6 d and 15–18 d were the periods when calluses appeared in large numbers. The level of endogenous IAA increased significantly during this period, indicating that this auxin has a promoting effect on cucumber callus induction. Interestingly, the endogenous abscisic acid (ABA) content changed dramatically during this process, suggesting that ABA is also involved in the process of cucumber regeneration [27]. Wang et al. [16] found that changes of endogenous hormones were different in different genotypes of cucumber, and only the CTK content was significantly positively correlated with the differentiation rate. The qPCR results showed that expression of *CYP735A* (involved in the regulation of cytokinin synthesis) is 18.08 times higher in highly differentiated than in poorly differentiated material. These results show that CTK is the critical factor affecting the regeneration activity of cucumber tissues [16].

Because different hormones have different effects, and different genotypes have different responses to exogenous hormones [16], researchers usually add various concentrations of different hormone combinations at each stage during the cucumber regeneration process to screen for optimal effects. In related reports, IAA, naphthalene acetic acid (NAA), gibberellic acid (GA), indole-3-butyric acid, 6-benzylamino purine (6-BA), and ZT have been used for the in vitro culture of cucumber tissues [11,37,38]. In the bud induction stage, the addition of exogenous auxin may promote callus formation rather than adventitious bud differentiation due to the high auxin content of cucumber itself, so auxin and its analogs are rarely used. Currently, the combination of 6-BA and ABA is frequently used to induce shoot regeneration in the process of cucumber transformation (Table 1). The combination of two hormones has been shown to effectively inhibit the formation of callus and directly produce bud clumps [39]. However, the molecular mechanism of ABA-stimulated shoot

induction and the interaction between ABA and 6-BA in the cucumber remain unknown. Some studies have speculated that ABA might be involved in regulating the water content of explants that do not become vitrified and/or activating stress tolerance genes, resulting in an increase in regeneration efficiency [18,40]. Interestingly, this effect appears to be seen only in cucumbers, whereas the addition of ABA to other cucurbit crops has been found to inhibit explant growth [40]. After the induction of adventitious buds is complete, they enter the rooting stage via elongation culture. Because such adventitious shoots have strong rooting ability, it is generally believed that only solid MS medium can give optimal rooting effects [23,24]. However, it was also observed that some materials did not take root well with MS medium alone [29]; therefore, auxin and its analogs were often added to the medium in some studies. These could promote the rooting of shoots and accelerate the rooting process of adventitious shoots [27,41]. In addition, thidiazuron is an exogenous hormone commonly used in the culture of unfertilized cucumber cells and ovules and plays a key role in inducing the stage of explant callus formation to somatic embryogenesis [42]. The plant regeneration rate was up to 79.3% when the ovary was cultured on medium supplemented with 0.06 mg·L^{-1} thidiazuron by Deng et al. [43].

Table 1. Application of plant hormones and $AgNO_3$ in the explant bud induction stage of different varieties of cucumber.

Variety	Explant Type	Treatment	Regeneration Frequency (%)	Reference
14–111	Cotyledon	3.0 mg·L^{-1} 6-BA + 2.0 mg·L^{-1} ABA + 1.0 mg·L^{-1} $AgNO_3$	86.7%	[38]
9330	Cotyledon	1.5 mg·L^{-1} 6-BA + 1.0 mg·L^{-1} ABA	96.7%	[18]
Xintai mici	Cotyledon	1.5 mg·L^{-1} 6-BA + 0.5 mg·L^{-1} ABA + 2.0 mg·L^{-1} $AgNO_3$	86.1%	[21]
Chuanlv No. 2	Cotyledon	1.5 mg·L^{-1} 6-BA + 0.5 mg·L^{-1} ABA	96.0%	[44]
	Cotyledon	3.0 mg·L^{-1} 6-BA + 0.5 mg·L^{-1} IAA	70.0%	[11]
Jinyan No. 4	Cotyledonary nodes	3.0 mg·L^{-1} 6-BA + 1.5 mg·L^{-1} ABA + 1.5 mg·L^{-1} $AgNO_3$	90.0%	[22]
S52	Cotyledonary nodes	3.0 mg·L^{-1} 6-BA + 1.5 mg·L^{-1} ABA + 1.5 mg·L^{-1} $AgNO_3$	90.0%	[30]
9330	Cotyledonary nodes	2.0 mg·L^{-1} 6-BA + 1.0 mg·L^{-1} ABA + 2.0 mg·L^{-1} $AgNO_3$	90.2%	[25]
Xintai mici	Cotyledonary nodes	0.5 mg·L^{-1} 6-BA + 1.0 mg·L^{-1} ABA	82.7%	[28]
Xintai mici	Cotyledonary nodes	1.5 mg·L^{-1} 6-BA	94.3%	[45]
Green Long	Cotyledonary nodes	1.5 mg·L^{-1} 6-BA + 4.5 mg·L^{-1} $AgNO_3$	100.0%	[10]
Gergana	Hypocotyl	1.0 mg L^{-1} BA	93.3%	[14]
Poinsett 76	Embryonal axis	4.44 µM 6-BA + 1.59 µM NAA	82.0%	[12]

2.4. Silver Nitrate ($AgNO_3$)

During tissue culture, explants will produce ethylene, which affects the in vitro culture. Mohiuddin et al. [46] demonstrated that $AgNO_3$ has an inhibitory effect on ethylene production and can significantly improve the induction rate of cucumber buds in vitro and the number of buds per explant. In ovary tissue culture, $AgNO_3$ can also promote embryogenesis and improve embryo yield [43]. Therefore, $AgNO_3$ has been widely used as an ethylene antagonist in the in vitro culture of cucumber material, and the optimal concentration of $AgNO_3$ used in relevant reports was mostly 0.5–2.0 mg·L^{-1} (Table 1).

3. Factors Influencing the Genetic Transformation of Cucumber

A. tumefaciens achieves genetic transformation by transferring the T-DNA containing the gene of interest into host cells. In this technique, *Agrobacterium*–plant interaction occurs when the bacteria are exposed to a large number of plant-derived chemicals, which include the routinely secreted chemicals, such as organic acids (pH 5.0–5.8) and other secondary metabolites, and the wound-releasing chemicals, such as phenolic compounds [5]. Subsequently, a series of related genes are expressed to direct the excision of T-DNA from the Ti plasmid, transfer to the host cell, integrate into the host genome, and be expressed in the host cell [5]. During *Agrobacterium*-mediated transformation, the transformation frequency is determined by several major factors including pre-culture and co-culture periods, *Agrobacterium* strain, acetosyringone (AS) concentration, bacterial cell density, duration of exposure to *Agrobacterium*, and selection system used.

3.1. Agrobacterium Strains

Different *Agrobacterium* strains containing a series of binary plant vectors have been applied in cucumber to obtain transgenic plants (Table 2). Because of the difference in the ability of *Agrobacterium* strains to infect different genotypes, the transformation efficiency (transformed shoots per explant) was often affected. Selvaraj et al. [6] demonstrated that *Agrobacterium* strains EHA105 and LBA4404 carrying the same construct showed different transformation abilities using cucumber cotyledons as explants, and the former strain exhibited an approximately threefold increase in transformation efficiency (21%) over the latter one (8.5%). This obvious difference in transformation efficiency by using different strains was also observed by Zheng [23]; thus, compared with GV3101, EHA105 has a higher conversion rate. The data in Table 2 show that EHA105, LBA1404, and GV3101 are frequently used in the genetic transformation of cucumber, and higher transformation efficiency always seems to be obtained with EHA105 (Table 2).

Table 2. Details of some successful reports on *Agrobacterium*-mediated transformation of cucumber.

Genotypes	Explant	*Agrobacterium* Strains	Plasmid Vectors	Transgene Constructs	Transformation Efficiency (%)	References
cv. Cengelköy	Cotyledon	EHA101	pGA482GG	nptII + GUS	16	[26]
cv. Poinsett76	Cotyledon	EHA105	pME504	CaMV35S::nptII + bar + GUS	1.6	[33]
cv. Poinsett76	Cotyledon	EHA105	pME508	CaMV35S::bar + sGFP-TYG NosP::nptII	21	[6]
cv. Shinhokusei 1	Cotyledon	EHA105	pIG121-Hm pIG-sGFP pGFP-S65C	NosP::nptII CaMV35S::GUS + HPT NosP::nptII CaMV35S::GUS+sGFP NosP::nptII CaMV35S::sGFP + HPT		[40]
cv. M8	Cotyledon node	EHA105	pCAMBIA1300-2A11-INH	2A11::INH	2	[47]
cv. CU2	Cotyledon	EHA105	pKCE402		1	[48]
cv. CU2	Cotyledon	EHA105	pHCG401/pKCG401-CsWIP1	CsU6–1::gRNA d35s::ZmCas9 + eGFP + Hgy	0.89	[49]
			pHCG401/pKCG401-CsVFB1		0.87	
			pHCG401/pKCG401-CsMLO8		0.25	
			pHCG401/pKCG401-CsGAD1		0.83	
cv. Nongcheng No. 3	Cotyledon	GV3101	Partgk-2		3.9	[50]
cv. Xintaimic	Cotyledon	GV3101	pCAMBIA2301s		2.89	[8]
cv.9930					4.13	
cv. Xintaimic	Cotyledon node	GV3101	pCAMBIA2300s	CaMV35S::nptII	8.1	[7]
cv. g1	Cotyledon node	GV3101	pCAMBIA2301-GUSA	GUS + nptII	2.33	[51]
cv. Cs0601	Cotyledon node	GV3101	pCAMBIA–1301	p35S::HYG	2.4	[41]
		LBA4404			0.5	
cv. Xintaimic	Cotyledon node	LBA4404	pBI121	35S::nptII + 35S::gusA	4.8	[30]
cv. Xintaimic	Cotyledon node	LBA4404	pBI121	NOS::nptII	4.8	[45]
cv. Shital	Leaf	LBA4404	pBI121	CaMV35S::GUS + NOS::npII		[9]
cv. poinsset76	Cotyledon	AGL1		35s::GU S + 35s::nptII	0.5	[18]
cv. Changchun-mic	Cotyledon node	C58	pGreen0029		1	[52]

3.2. Pre-Culture Period

Pre-culture refers to the process of culturing explants in differentiation medium before *Agrobacterium* infection. Generally, pre-culture promotes cell division and adjusts the physiological condition of explants, which is conducive to the integration of exogenous T-DNA into explants. If the pre-culture time is too short, it is difficult for the explants to reach the optimal physiological state, and it may even be difficult to differentiate into regenerated plants [53]. However, the pre-incubation time should not be too long, otherwise a protective layer will be formed outside the wound, which will reduce the infection efficiency, or untransformed cells will form cell clusters, which will increase the resistance to the selection agent and inhibit the differentiation of transformed cells [23]. Wang et al. [39] found that explants pre-cultured for 1 day grew more resistant shoots than explants used for direct infection, and the rate of resistant shoots increased from 11.1% to 27.3%; however, as the pre-culture time was prolonged, the cell division ability was weakened, and the transformation rate also began to decline. Similar conclusions were obtained by Sun et al. [41]: with the progress of pre-culture, the bud induction rate first increased and then decreased, and 2 days was very effective in producing the highest cucumber regeneration frequency and

number of shoots per explant (90.01% and 1.5, respectively). Fan et al. [54] reported that compared with 1–2 d of pre-cultivation, a higher number of regenerated buds and resistant buds could be obtained in 3–7 d, considering that an excessive pre-culture time will lead to difficulties in later positive screening. A duration of 3 d was finally selected as the best pre-culture time. Different genotypes selected in the study, different environmental conditions during pre-culture, or different concentrations and ratios of hormones added to the pre-culture medium can affect the time required for the explants to reach the optimal physiological state, so that the optimal pre-incubation time chosen in the study also varied: 1–2 d is most commonly used in relevant reports. In addition, in the process of tissue culture, the peroxidase activity of explants was significantly positively correlated with the frequency of plant regeneration, while culture in the dark can increase the peroxidase activity of explants during the induction process and keep it at a high level. Therefore, pre-culture is often carried out in the dark [55].

3.3. Bacterial Cell Density and Length of Infection

Cucumber transformation efficiency is significantly affected by bacterial cell density and the duration of infection. Wei et al. [56] investigated the effect of bacterial cell density on the transformation of unfertilized ovary and found that lower density with an optical density at 600 nm (OD_{600}) of 0.3 was beneficial to embryogenesis, whereas higher density (OD_{600} = 0.6) decreased infection efficiency. The study also explored the optimal infection and suitable co-cultivation times, which could help to establish a genetic transformation system for unfertilized ovarian cells. Similar results were also obtained in several different cucumber genotypes by Fan et al. [54] and Yang et al. [44] in cotyledons: with an increase in bacterial cell density, the *Agrobacterium* grew too much, and the explants browned and died. However, other results showed that lower density was not effective, and on the basis of the plant regeneration capacity or transformation efficiency, OD_{600} = 0.6–0.8 was recommended [18,53]. At this time, the strain is in the logarithmic growth phase and has high infective activity. It is worth mentioning that the bacterial cell density measured by the OD value method contains both live bacteria and dead bacteria, which cannot represent the true viability of *Agrobacterium;* thus, the optimal bacterial density needs to be further explored [52].

The inoculation time will be affected by selected genotype, bacterial cell density, and *Agrobacterium* viability, so resistant shoots can be obtained within minutes or tens of minutes [45,49]. However, in general, 10–20 min of inoculation time has usually been used for cucumber genetic transformation [47,50,57]. In addition, certain measures can be taken to promote the contact between *Agrobacterium* and explants. Silwet-76 as a surfactant was added to an *Agrobacterium* suspension. This effectively increases the rate of β-glucuronidase (GUS) staining as well as the differentiation of shoots [51]. Rajagopalan and Perl-Treves [33] demonstrated that explants pricked with a needle underwent transient transformation more often than controls, showing that such wounding treatment was effective in improving transformation efficiency. A physical method using vacuum infiltration was developed to enhance *Agrobacterium* infection by Nanasato et al. [40]. The average transformation efficiency was as high as 11.9 ± 3.5% in four replicate transformation experiments. Successful transformation also has been achieved by Zhang et al. [21]: explants were placed in a beaker with *Agrobacterium* suspension and vacuum infiltrated twice for 5 min, then moved into a shaker for an additional 5 min, giving a final transformation frequency of 26%. However, the use of vacuum pumps is cumbersome, hindering the promotion of this physical method. The procedure was greatly simplified by Hu et al. [49], in that the vacuum pump was replaced with a simple syringe, and green fluorescent protein detection showed that the regeneration site was not consistent with the infection site. Compared with pure immersion infection, vacuum negative pressure can significantly improve the infection depth of *Agrobacterium* so that it can reach the regeneration site inside the explants [49].

3.4. Conditions of Co-Cultivation

After *Agrobacterium* infection, the co-cultivation stage is entered, the purpose of which is to facilitate the contact of *Agrobacterium* with the explant, as well as the efficient insertion of a foreign gene. Shorter co-cultivation times will reduce the number of foreign genes inserted into the plant genome, while longer times will overgrow residual *Agrobacterium* on explants, harming the plant cells [56]. Co-cultivation for 2–3 d has been used optimally in many studies [8,50]. Growth of explants, the optimal temperature for *Agrobacterium* infection, T-DNA transfer, and interaction with the co-cultivation time should be fully considered when studying the co-cultivation temperature. It was found that 22–26 °C was a suitable temperature for co-cultivation [44,52]. In a study of co-culture pH levels, it was demonstrated that lower pH values can enhance the induction effect of acetosyringone (AS), which is beneficial for the activation and expression of the *Agrobacterium* virulence (*Vir*) genes [58]. Ning et al. [59] suggested that the combination of pH 5.2 and 100 µmol/L AS was the optimal co-culture condition for cucumber transformation. Appropriate light condition during co-cultivation was tested by Rajagopalan and Perl-Treves [33]. In that study, explants were cultured in complete darkness or under a 16/8 h light/dark photoperiod. Although there was no obvious difference in transient GUS expression, a higher survival rate and regeneration frequency were observed in light-grown explants. Similar results were obtained by Fan et al. [54], in that more resistant shoots were obtained in cotyledon nodes cultivated under light. However, it is generally believed that co-cultivation should be carried out under dark conditions, which can reduce the overflow of phenolic substances from explants [60]. Because bacteriostatic agents are generally not added to the co-culture medium, the explants can contain a high content of *Agrobacterium* after co-cultivation. Therefore, filter paper wicks were used to isolate solid media and *Agrobacterium* [40]. In addition, the bacterial elimination treatment was performed before the culture screening to reduce the contamination of the medium and explants by *Agrobacterium* in later stages [18]. Chang et al. [29] found that when explants were washed three times with sterile water, the infection rate was low, and the explants grew well. Chen et al. [8] demonstrated that adding a certain concentration of carbenicillin (Carb) to sterile water has a better bacterial elimination effect, but it should be noted that the Carb needs to be rinsed off subsequently.

3.5. Acetosyringone (AS) Concentration

The addition of AS into *Agrobacterium* suspensions and pre-culture and co-culture media can enhance the genetic transformation of cucumber because acetosyringone can induce the activation and expression of virulence genes in *Agrobacterium*, affecting gene transformation efficiency by promoting T-DNA transfer. The use of acetosyringone was comprehensively affected by plant species, strains, and pH values, resulting in differences in the optimal concentration of acetosyringone found in different studies [43]. The lowest concentration used in cucumber was 20 µmol/L [25], whereas the highest was 500 µmol/L [47]. In general, 50–150 µmol/L concentrations have been used frequently in most reports [3,59]. In addition, in recent years, the strong antioxidant α-lipoic acid has been used as a new inducer in cucumber, and this can synergize with AS to improve transformation efficiency. The commonly used concentration in cucumber is 50–100 µmol/L [3]. However, because AS and α-lipoic acid can be toxic to the explants, and this can be affected by the medium's pH, type of explants, and co-cultivation temperature, the specific addition method and concentration need to be confirmed experimentally [3].

3.6. Selection Markers

When explants are inoculated with *Agrobacterium*, only the cells near the incision are infected easily. In the absence of selection pressure, some untransformed cells might also initiate differentiation. Therefore, antibiotics are often added to the differentiation medium as selection agents to kill or inhibit untransformed cells. Kanamycin, hygromycin, and phosphinothricin are the most widely employed for cucumber. As the tolerance of explants to antibiotics depends on genotype and culture stage, before transformation,

the optimal concentrations for shoot and root induction have been screened by adding different concentrations of antibiotics into the explant culture medium [8,45]. Normally, 50–150 mg/L kanamycin, 2–25 mg/L hygromycin, and 2–6 mg/L phosphinothricin are applied for cucumber transformation [51]. At an initial stage of induction, the metabolism of transformed cells is not yet fully normalized, so they are easily killed by high concentrations of antibiotics before budding. Therefore, a more effective way to conduct positive screening is by establishing a concentration gradient over time. At the beginning of differentiation, a low concentration of antibiotics is added, and when the shoot primordium is fully differentiated, a higher concentration is employed to eliminate false-positive plants [9,28,33]. To inhibit *Agrobacterium* contamination, bacteriostatic agents are often used in the selection medium. Apart from effectively inhibiting the growth of *Agrobacterium*, antibiotics should also be chosen so as not to affect plant regeneration as much as possible [23]. According to the bacteriostatic effect, contamination rate, and germination rate, 300–500 mg/L cefotaxime sodium (Cef), carbenicillin (Carb), and timentin (Tim) were applied [29,30,53]. Tim has the best antibacterial effect and the longest duration relative to Cef and Carb, but it also has the disadvantage of high cost [7]. It is inevitable that the growth of plants and the regeneration of buds will be affected by antibiotics. Therefore, non-resistance marker genes such as green fluorescent protein (GFP) and GUS have been applied to the genetic transformation of cucumber. The transient expression rate of GUS is often used to select the best conditions for infection [51], pre-culture [30], and co-culture [33]. Unfortunately, detection with a GUS assay was destructive, and identification of stable transformation events took at least 2–4 weeks [33]. In contrast, when the *GFP* gene was used, the labeled proteins could be observed in vivo by fluorescence microscopy. The early detection of GFP expression has greatly helped in the rapid discrimination of transformants, escapes, and chimeras [6]. Hu et al. [49] observed faint fluorescence at 3 days post-infection under a stereoscopic fluorescence microscope. In addition, non-antibiotic selection markers have been used in cucumber transgenesis. He et al. [61] reported a mannose selection system, in which mannose can only be utilized as a carbon source by cucumber cells possessing phosphomannose isomerase, finding that there were few false positives and the highest transformation efficiency when the concentrations of mannose and sucrose in the medium were both 10 g/L.

4. Current Status of the Application of Cucumber Genetic Transformation

Transgenic technology is potentially an important means for cucumber variety improvement (Figure 3). Using cucumber transformation to alter gene expression, complement mutations and knockout genes are powerful tools for understanding the functions of genes of interest in cucumber [62,63]. As illustrated below, research on other plants, especially *Arabidopsis*, has made important contributions to the identification of genes controlling cucumber development. The functions of these genes need to be investigated in relation to the specific features of cucumber development. Overexpression of target genes and RNA interference (RNAi) mechanisms are the main biotechnological tools exploited to regulate the expression levels of target genes. In addition, new breeding techniques such as genome editing mediated by clustered regularly interspaced short palindromic repeats (CRISPR) with CRISPR-associated protein 9 (Cas9) have been developed. Unlike RNA interference (binding siRNA with the mRNA of the target gene to reduce the level of gene expression), this is a precise tool to introduce targeted mutations strategically in the host genome [64]. This technique was soon applied to develop broad-spectrum antiviral [65], dwarf [48], and gynoecious cucumber lines [49].

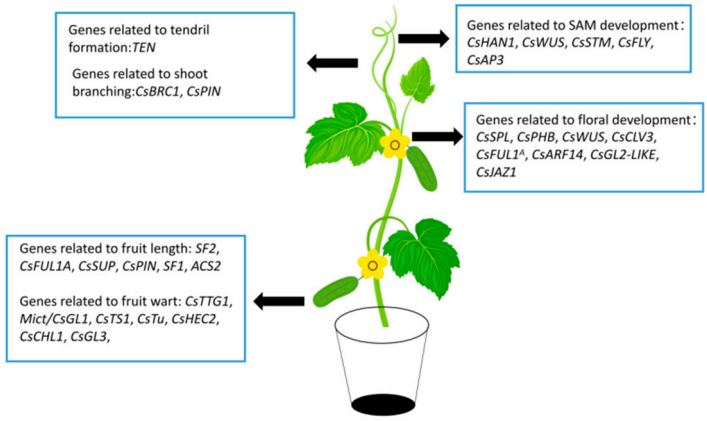

Figure 3. Illustration of agronomic trait-related genes in cucumber plants.

4.1. Genes Associated with Shoot Apical Meristem (SAM) Development

All the organs of cucumber including leaves, flowers, and stems are derived from the SAM. *HANABA TARANU (HAN)* codes for a transcription factor that participates in the regulation of SAM organization and flower organ development in *Arabidopsis* [66]. In cucumber, elevated expression of *CsHAN1* produced delayed growth, reduced flower buds, and lobed leaves. Interestingly, a *CsHAN1-RNAi* line exhibited similar phenotypes with delayed SAM development versus control plants. Furthermore, CsHAN1 may interact physically with CsWUS and CsSTM, the counterparts of key regulators of SAM development in *Arabidopsis* [66]. In addition, *LEAFY (LFY)* and its homologs have been shown to promote flower development and branching [67]. Therefore, *CsLFY* was also cloned and functionally analyzed in cucumber [67]. The *CsLFY-RNAi* line displayed adventitious shoots with few leaves. Subsequently, yeast two-hybrid screening and a bimolecular fluorescence complementation assay revealed that CsFLY interacts directly with CsWUS to regulate shoot meristem maintenance in cucumber [67].

4.2. Genes Related to Floral Development

Anthers and ovules play important roles in crop fertilization and fruit production [68]. In *Arabidopsis*, the *SPOROCYTELESS (SPL)/NOZZLE (NZZ)* gene has been shown to play essential roles in anther and ovule development. A recent study showed that the *SPOROCYTELESS (SPL)/NOZZLE (NZZ)* homolog *CsSPL* was extremely weakly expressed in a cucumber mutant, with severely defective anther and ovule development [68]. Reducing the expression of *CsSPL* by RNAi led to malformed pollen, suppressed ovule development, and reduced male and female fertility. Further, biochemical analyses indicated that CsSPL acts as a linker between between CsPHB, which is a key regulatory gene expressed in the nucellus, and CsWUS [68]. The number of carpels is related to the shape, size, and quality of the cucumber fruit [69]. Moreover, the *CLV3* and *WUS* genes are the key regulators responsible for locule number increases in tomato [69]. Che et al. [69] reported that both *CsCLV3-RNAi* and overexpression of *CsWUS* resulted in increased petal and carpel numbers, suggesting that *CsCLV3* and *CsWUS* function as negative and positive regulators for carpel number variation, respectively. Cucumbers are naturally dioecious, with male and female flowers, and are a model plant for the study of sex differentiation. However, little research has been done on the regulatory mechanism of its flowering [69]. Knockdown of the cucumber homolog of the *AtGL2* gene, involved in epidermal cell determination in *Arabidopsis*, delayed male flower flowering, reduced pollen and seed vigor, and caused partial male sterility. Further transcriptome analysis and protein–protein interaction assays showed that CsGL2-LIKE directly interacts with a jasmonate ZIM domain protein CsJAZ1 in regulating male flowering in cucumbers [70].

4.3. Genes Associated with Fruit Development

4.3.1. Fruit Length

Fruit size and shape are important agronomic traits that are associated with quality. In *Arabidopsis*, the histidine deacetylase complex gene HDC1 regulates ABA sensitivity and promotes increases in organ size [71]. A recent study identified recessive allelic variation in a cucumber *HDC1* homolog gene *Short Fruit2 (SF2)*. This mutation significantly repressed fruit elongation [71]. Homozygous complemented plants were constructed by transferring the vector carrying the SF2 coding region together with 995 bp of the promoter into the *sf2* mutant, and these plants displayed increased cell numbers and fruit length. Moreover, *SF2* directly targets cytokinin and polyamine biosynthesis to regulate cell proliferation [71]. Apart from that, two alleles of the *FRUITFULL (FUL)*-like MADS-box gene *CsFUL1* with a Q–K single nucleotide polymorphism associated with variation in fruit length were identified among 150 cucumber lines [72]. Overexpression of $CsFUL1^A$ resulted in reduced fruit length compared with controls, whereas knockdown of $CsFUL1^A$ by RNAi increased fruit length. Further transcriptome analysis and biochemical data revealed that $CsFUL1^A$ inhibits *CsSUP*-mediated cell division and expansion as well as *CsPIN*-mediated auxin transport by binding to their promoter regions [72]. Ethylene plays an essential role in cucurbit fruit development. Xin et al. [73] reported the ethylene regulation mechanism of cucumber fruit development; knockout of *SF1*—encoding a cucurbit-specific RING-type E3 ligase that regulates ethylene dosage—resulted in overproduction of ethylene, whereas knockdown of *ACS2*—encoding a rate-limiting enzyme for ethylene biosynthesis—led to reduced ethylene production. However, both mutants produced shortened fruits, suggesting a possible dose-dependent effect of ethylene on cucumber fruit length regulation. Further biochemical analyses indicated that SF1 specifically interacts with ACS2 to achieve precise control of ethylene dosage in cucumber fruits [73].

4.3.2. Fruit Wart

The cucumber wart consists of fruit trichomes (spines) and underlying tubercules. This is an important agronomic trait that directly affects fruit appearance and market value [74]. The WD-repeat protein *TTG1* in *Arabidopsis* was shown to regulate trichome differentiation. Moreover, the cucumber WD-repeat homolog *CsTTG1* is specifically expressed in the epidermis of cucumber ovaries [74]. Silencing *CsTTG1* by RNAi resulted in reduced fruit spines in cucumber, whereas overexpression of *CsTTG1* increased the density of fruit bloom trichomes and spines. Further molecular studies and biochemical analyses indicated that CsTTG1 interacts with the trichome formation regulator Mict/CsGL1 to regulate wart formation [74]. In *Arabidopsis*, *TS1* encodes oleosin proteins, which are plant-specific factors that regulate oil body size. Unlike its homologous *Arabidopsis* gene, *CsTS1* functions in regulating fruit tubercule development [75]. Analysis by RT-qPCR revealed that it is highly expressed in the tubercules of fruit. Blocking the expression of *CsTS1* by RNAi led to smaller tubercules than controls, whereas elevated expression of *CsTS1* led to larger tubercules. Further studies indicated that as a positive regulator, the transcription factor CsTu might bind directly to the promoter of *CsTS1* to determine fruit tubercule formation in cucumber [75]. The basic helix-loop helix (bHLH) gene *HECATE2* is important for female reproductive tissue formation in *Arabidopsis* [76]. Its cucumber homolog *CsHEC2* is highly expressed in cucumber fruit peel including spines and tubercules. Knockout of *CsHEC2* resulted in a significant decrease in the density of spiny nodules on the fruit surface and a decrease in cytokinin accumulation, whereas overexpression of *CsHEC2* produced the opposite results. Further molecular and biochemical studies indicated that *CsHEC2* acts as a key cofactor for *CsGL3* and *CsTu* to regulate wart formation via *CsCHL1*, which codes for an enzyme involved in CTK biosynthesis [76].

4.4. Other Genes

Shoot branching directly affects plant structure and yield. The axillary branches need to be removed manually to promote crop yield and quality in actual production [77].

The TEOSINTE BRANCHED1/CYCLOIDEA/PCF(TCP) gene family is an integrator of multiple internal and external signals that act inside the axillary buds to suppress shoot branching in multiple species. Moreover, *CsBRC1* (the homologous gene of the TEOSINTE BRANCHED1/CYCLOIDEA/PCF(TCP) family of genes) is specifically expressed in axillary buds and is negatively correlated with branching [77]. Knockdown of *CsBRC1* by RNAi promoted lateral shoot growth and reduced auxin accumulation. Further molecular experiments demonstrated that *CsBRC1* inhibited the growth of cucumber axillary buds by inhibiting the activity of the auxin transporter PIN3 (PIN-FORMED3). This research could help to develop cucumber varieties with varying degrees of shoot branching [77]. The cucumber tendril identity gene *TEN* also belongs to the TCP gene family of transcription factors and regulates cucumber tendril development [78]. To knock out the *TEN* gene and further investigate functions associated with *TEN*, the CRISPR/Cas9 system was used to target amino acids 1–121 in the *N* terminus of the TEN protein. Some *TEN* gene-edited plants exhibited a complete transformation of tendrils into lateral branches, while others showed largely unaffected tendril morphology but greatly reduced climbing ability. Moreover, *TEN* directly regulates ethylene synthesis to control tendril morphology and climbing [78].

5. Conclusions and Future Perspectives

Since the advent of the cucumber genetic transformation, numerous researchers have been attracted to this topic, and therefore the transgenic technology has been improved and developed continuously. At present, transgenic systems of some cucumber lines have been established, but the efficiency of genetic transformation is universally low. Thus, the lowest reported transformation efficiency was 0.1% [55], while the highest was 26% [21]. This finding has many reasons. First, the few cucumber lines that have been used to obtain transgenic plants were selected easily, which has limited the use of some new varieties. Second, the concentration of antibiotics added to the screening medium has been too high, so some transformed cells have been killed at an early stage of differentiation. Third, the position of regeneration buds differs from that of *Agrobacterium* infection. Fourth, there are few studies on environmental factors such as culture temperature, pH of the medium, light conditions, air conditions, and their interactions. Last, there has been little research on some details, such as types of basal medium, curing agents, organic additives, antioxidants, and surfactants.

In most of the existing reports, a genotype with a higher regeneration rate was selected first, and many parameters affecting genetic transformation efficiency, such as co-cultivation conditions, *Agrobacterium* strains, and bacterial cell density, were optimized gradually. Finally, excellent transformation systems suitable for certain genotypes have been established. In summary, most studies have focused on using cotyledons and cotyledon nodes as explants, adding different concentrations of 6-BA, ABA, and $AgNO_3$; causing *Agrobacterium* infection using soaking or vacuum infiltration; and obtaining transgenic plants by direct bud regeneration. In particular, although the use of cotyledon nodes has become more frequent in recent years, cotyledons remain the most efficient explants to obtain transgenic cucumber lines, and the transformation efficiency has been up to 21%. In addition, vacuum infiltration is gradually replacing traditional immersion infection. This is a trend that will improve transformation conditions to achieve high frequency for cotyledons mediated by *Agrobacterium* strain EHA105 under vacuum.

In recent years, the method of using nanomaterials to deliver biomolecules into plants has gradually become well known. DNA has been delivered successfully to spinach, tobacco, watercress, and *Arabidopsis* using chitosan-wrapped single-walled carbon nanotubes, suggesting that this could be a general mechanism applicable to different plants [79]. In addition, it was demonstrated that nanoparticles can also carry short interfering (si)RNA into plants as a carrier to exert the gene silencing effect, and delivery of siRNA does not seem to require the nanocarriers to enter plant cells, but only needs them to be released in the apoplast to function [80]. It is foreseeable that with the application of nanotechnology in

cucumber in the future, the in-depth study of safety screening markers, and the continuous optimization of CRISPR/Cas9 technology, the problems currently plaguing researchers will be solved gradually.

Genomic transformation is an important tool for exploring the relationship between gene function and important agronomic traits of cucumber. Using overexpression and RNAi and gene editing technology, the molecular mechanisms of some important agronomic traits have been unraveled. To meet people's growing demand for high-quality, nutritious, and safe cucumber products, research on gene mining and regulatory network analysis with important breeding value should be increased in the future.

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