Programa de Doctorado en Bioingeniería

TESIS DOCTORAL

Development of tools for genetic transformation and tissue regeneration in plants

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INFORMAN:

Que Dña. Olha Yaroshko ha realizado bajo nuestra supervisión el trabajo titulado **"Development of tools for genetic transformation and tissue regeneration in plants"** conforme a los términos y condiciones definidos en su Plan de Investigación y de acuerdo al Código de Buenas Prácticas de la Universidad Miguel Hernández de Elche, cumpliendo los objetivos previstos de forma satisfactoria para su defensa pública como tesis doctoral.

Lo que firmamos para los efectos oportunos, en Elche.

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LIST OF ABBREVIATIONS

°C	degree Celsius
A. caudatus	*Amaranthus caudatus*
A. cruentus	*Amaranthus cruentus*
A. rhizogenes	*Agrobacterium rhizogenes*
A. thaliana	*Arabidopsis thaliana*
A. tumefaciens	*Agrobacterium tumefaciens*
bar	phosphinothricin N-acetyltransferase
CaMV	cauliflower mosaic virus
cm	centimetre
cm^2	square centimetre
CRISPR/Cas	clustered regularly interspaced short palindromic repeats/CRISPR-associated)
cv.	cultivar
d	*dwarf*
DNA	deoxyribonucleic acid
DsRED	RED FLUORESCENT PROTEIN
E. coli	*Escherichia coli*
ej-2^w	*enhancer of jointless*
GFP	GREEN FLUORESCENT PROTEIN
hph	hygromycin phosphotransferase
i.e.	id est (that is)
I	*Immunity to fusarium wilt*
LB	left border
mg/L	miligrams per-litre
mm	milimetre
mM	milimolar
mnt	*miniature*
MPa	megapascal
nos	nopaline synthase
nptII	neomycin phosphotransferase
obv	*obscuravenosa*
ocs	octopine synthase
RB	right border
RNA	ribonucleic acid
S. lycopersicum	*Solanum lycopersicum*
S. pimpinellifolium	*Solanum pimpinellifolium*

Sm	*Stemphylium resistance*
sp.	species
sp	*self-pruning*
spp.	species (more than one)
T-DNA	transferred-DNA
Ti plasmid	tumor-inducing plasmid
u	uniform ripening
uidA	β-glucuronidase A enzyme
var	variety
Ve	*Verticillium resistance*
X-gluc	5-bromo-4-chloro-3-indolyl-β-D-glucuronide

ABSTRACT

Climate change is negatively affecting agricultural production. Therefore, it is crucial to develop new plants that are tolerant or resistant to abiotic and biotic stresses. Biotechnological tools, such as genetic modification and genome editing, can be used to rapidly create plants with new traits. Plant biotechnology utilizes gene editing tools such as zinc finger nucleases, transcription activator-like effector nucleases, and clustered regularly spaced short palindromic repeats (CRISPR)/CRISPR-associated (CRISPR/Cas) proteins. While plant genome editing is a new field, most editing systems are based on established genetic transformation methods. The delivery methods for the editing tools, such as Agrobacterium-mediated delivery, biolistics, and polyethylene glycol-mediated transformation, are the same as those used for classic genetic transformation. To achieve successful editing, intermediate steps such as plant regeneration and transformation must be optimized beforehand.

This thesis aims to determine the optimal conditions for *Agrobacterium tumefaciens*-mediated transformation of *Amaranthus caudatus* cultivars and establish a regeneration methodology for *Solanum lycopersicum* and heirloom tomato genotypes. An effective methodology for transforming *Amaranthus caudatus* cultivars, Karmin and Helios, was established, with results obtained within four days of the experiment's start due to the speed of the transformation method. The procedure for obtaining and regenerating calli was developed for three commercial tomato varieties and three wild accessions. Optimal conditions for obtaining the maximum

number of regenerated shoots and callus formation were evaluated for all the tomatoes studied. Our method allows for obtaining initial results in less than two weeks. The factors that significantly influence the percentage of regeneration and callus formation were assessed.

RESUMEN GLOBAL

El cambio climático está afectando negativamente a la producción agrícola. Por eso es crucial desarrollar nuevas plantas tolerantes o resistentes al estrés abiótico y biótico. Las herramientas biotecnológicas, como la modificación genética y la edición del genoma, pueden utilizarse para crear rápidamente plantas con nuevos rasgos. La biotecnología vegetal utiliza herramientas de edición genética como las nucleasas de dedos de zinc, las nucleasas efectoras similares a activadores de la transcripción y las proteínas asociadas a repeticiones palindrómicas cortas agrupadas regularmente espaciadas (CRISPR)/Cas. Aunque la edición del genoma vegetal es un campo nuevo, la mayoría de los sistemas de edición se basan en métodos establecidos de transformación genética. Los métodos de administración de las herramientas de edición, como la administración mediada por *Agrobacterium* spp., la biolística y la transformación mediada por polietilenglicol, son los mismos que los utilizados para la transformación genética clásica. Para que la edición genética tenga éxito, es necesario optimizar previamente los pasos intermedios, como la regeneración de la planta y la transformación.

Esta tesis pretende determinar las condiciones óptimas para la transformación mediada por *Agrobacterium tumefaciens* de cultivares de *Amaranthus caudatus* y establecer una metodología de regeneración para genotipos de *Solanum lycopersicum*. Se estableció una metodología eficaz para transformar los cultivares de *Amaranthus caudatus*, Karmin y Helios, con resultados obtenidos a los cuatro días del inicio del experimento debido a la rapidez del método de transformación. En esta tesis se optimizó el

procedimiento de obtención y regeneración de callos para tres variedades comerciales de tomate y tres entradas silvestres. Se evaluaron las condiciones óptimas para obtener el máximo número de brotes regenerados y la formación de callo para todos los tomates estudiados. Nuestro método permite obtener resultados iniciales en menos de dos semanas. Se evaluaron también los factores que influyen significativamente en el porcentaje de regeneración y formación de callo.

1. GENERAL INTRODUCTION

1.1 *Agrobacterium*-Mediated Plant Transformation

Agrobacterium tumefaciens, also known as *Rhizobium radiobacter*, is a Gram-negative soil bacterium. It can insert part of its plasmid DNA into the nuclear genome of plant cells, causing them to proliferate abnormally and resulting in the formation of crown galls in many dicotyledonous plant species [1]. Additionally, *A. tumefaciens* controls the physiological processes of plants to synthesize specific compounds called opines, which are used by the bacteria as important sources of energy, carbon, and nitrogen [1]. The virulence of *A. tumefaciens* strains is dependent on the presence of the tumor-inducing (Ti) plasmid. The Ti plasmid contains all the necessary genes for infection, transfer, and integration of the T-DNA (transferred-DNA) region into the chromosomes of plants [2].

Ti plasmids from various strains of *A. tumefaciens* share common characteristics based on the primary functions of their genes [3]. The *ori* and *tra* regions are responsible for replication and transfer of the Ti plasmid to other bacteria during conjugation, respectively. The *vir* region contains the genes responsible for the pathogenicity of *A. tumefaciens* and the transfer of the T-DNA to plant cells. Approximately 25 different *vir* genes are grouped into operons designated *virA* to *virH* .[3]. Additionally, the *occ* and *noc* regions in the octopine and nopaline type Ti plasmids, respectively, are responsible for the catabolism of octopine and nopaline. The T-DNA region of the Ti plasmid is the only DNA sequence that is transferred into the plant cell and contains two types of genes: oncogenic genes that encode enzymes involved in the synthesis of auxins and cytokinins, causing transfected plant cells to proliferate [4], and opine biosynthetic genes responsible for the synthesis of manopin (*mas1* and *mas2*), agropin (*ags*), octopine (*ocs*), or nopaline (*nos*), which will be produced and secreted by transfected plant cells [5]. The T-DNA region is flanked

by specific DNA sequences, named as left border (LB) and right border (RB), which play a key role in T-DNA transfer and integration. Replacing the naturally occurring tumor-inducing genes and genes encoding opines in the T-DNA with other genes of interest, *A. tumefaciens* can be used as an efficient delivery system for genetic engineering of plant cells [6].

Another species of *Agrobacterium* commonly used in genetic engineering is *Agrobacterium rhizogenes* (also known as *Rhizobium rhizogenes*), which induces the 'hairy root' syndrome. Virulent strains of *A. rhizogenes* contain the root inducer (Ri) plasmid that has two T-DNA regions, TL-DNA and TR-DNA, which can be independently transferred to the nuclear genome of infected plant cells [7]. The of four *rol* genes (*rolA* to *rolD*) of the TL-DNA after its integration into the plant genome stimulates the formation of hairy roots [8,9]. The TR-DNA carries the genes responsible for opine synthesis, and the *aux1* and *aux2* genes encoding enzymes for auxin biosynthesis [9]. The process involves inoculating excised plant tissues (*i.e.*, explants) with virulent *A. rhizogenes* for varying periods. *A. rhizogenes* infects and induces hairy root growth in many plant species and is commonly used to create transgenic roots for biotechnological applications [1].

The interaction between *A. tumefaciens* and plant cells involves a complex series of signaling events [10]. When plant cells are wounded, they release phenolic compounds such as acetosyringone, syringaldehyde, and acetovanillone, as well as sugars like glucose, glucuronic acid, and arabinose. These compounds are as chemoattractants towards nearby agrobacteria, which can physically attach themselves to plant cell walls through newly synthesized cellulose fibers and cyclic glucans [3,10] . These signals derived from damaged plant cells also activate the expression of bacterial *virA* and *virG* genes, amplifying the transcriptional response of *vir* gene expression in the Ti plasmid [10]. The VirD1-VirD2 complex binds to LB and RB sequences of the T-DNA and catalyzes the nicking of a single-stranded T-DNA [11] that is

translocated towards the plant cell cytoplasm through a type IV secretion system localized in the cell envelope and extracellular T-pili [12].

Upon entering the cytoplasm of the plant cell, the T-DNA is shielded by the VirE2 protein to prevent degradation by endogenous nucleases. The VirD2 protein, which is bound to the 5' end of the T-DNA, facilitates the nuclear translocation of the T-DNA-VirE/D complex. Once inside the nucleus, VirE2 dissociates, and the single-stranded T-DNA is integrated into the plant genome through illegitimate recombination due to the low homology between endogenous DNA and the T-DNA sequences. Based on a recent model [13] double-stranded T-DNA synthesis may be facilitated by an endogenous DNA polymerase. This results in the dissociation of the VirD2 protein from the T-DNA. The plant genome integrates the double-strand T-DNA sequence through the non-homologous end-joining mechanism, recognizing it as genomic double-strand breaks. This integration is a rare event that can occur randomly at any site on the plant chromosome.

Marker or selection genes are utilized to identify plant cells and tissues that stably incorporate T-DNA into their genome [14]. These genes encode protein products that act in a dominant manner [14]. Selecting agents must not have any negative effects on transformed cells and should be effective at low concentrations. The expression of these genes is controlled by promoters of plant, bacterial, or viral origin, which usually have constitutive expression and are expressed in all plant tissues and organs [15]. Most selection genes are of bacterial origin [14,15]. The *nptII* gene of *Escherichia coli* encodes a neomycin phosphotransferase, which confers resistance to the antibiotic kanamycin [14,15]. This gene is highly effective in selecting transformed cells in many dicotyledonous species, but less so in monocotyledonous plants [14]. The *hph* gene of *E. coli* encodes a hygromycin phosphotransferase that provides resistance to hygromycin [14,15]. The *bar* gene, derived from *Streptomyces hygroscopicus*, is a commonly used selection marker. It encodes

a phosphinothricin N-acetyltransferase [14,15], which confers resistance to herbicides containing phosphinothricin. These herbicides inhibit glutamine synthetase activity and, consequently prevent glutamine biosynthesis and protein synthesis in plants [13,14]. Additional marker genes are employed to identify transformed plant cells and tissues through chemiluminescence, fluorimetry, or histochemical methods, and are named reporter genes [14,15]. This group includes genes encoding fluorescent proteins, such as the GREEN FLUORESCENT PROTEIN (GFP) from the jellyfish *Aequoria victoria* or the RED FLUORESCENT PROTEIN from *Discosoma* sp. (DsRED), or those like the *uidA* gene from *E. coli* and several *Lactobacillus* species that encodes the enzyme β-glucuronidase A [16,17]. The enzymatic activity of β-glucuronidase A can be detected by incubating the transformed tissue with a substrate that changes colour from colourless to bluish after enzymatic modification [15].

Several factors can affect the outcome of transformation experiments, including the *Agrobacterium* spp. strain, the type and age of the explants used for transformation, the composition of the culture medium, and the presence of an effective antibiotic concentration for eliminating bacteria after co-culture [18]. Hairy roots can be obtained from various plant tissues, such as roots, stems, or leaves [19–22]. *Agrobacterium*-mediated transformation is ineffective for plant species or genotypes with a low capacity for tissue regeneration. Thus, the first step before transformation is to establish a complete and effective seedling regeneration protocol from the tissue explants used for *Agrobacterium* transformation.

1.1.1 Transient Gene Expression

Transient gene expression refers to the temporary expression of genes that are introduced into eukaryotic cells through chemical, physical, or biological approaches [23]. In plants, there are various systems that can be used for transient gene expression [24], such as protoplasts [25,26], cell

suspension cultures [27], tissue explants [26,28,29], calli [30], and complete plants [31]. In all these cases, the genetic material that has been incorporated into plant cells is usually not transmitted to the offspring during the sexual reproduction of the plants derived from these transformed tissues [30]. Transient gene expression accelerates research by allowing in vivo functional analysis of the gene of interest within two to ten days after the introduction of the transgene to plant cells [24,28,30]. Transient expression of the transgene enables evaluation of the localization of the products encoded by the gene of interest, as well as the study of protein-protein interactions in which they participate [32,33].

One of the most effective methods for transiently expressing transgenes in plant tissues is using *A. tumefaciens*. The method involves physically penetrating plant tissues with a solution containing agrobacteria using high pressure generated by vacuum or syringes, with or without needles [23]. These are dubbed as vacuum infiltration, agroinjection and agroinfiltration, respectively. Additional advantages of this technology are its simplicity and speed, which allows for the infiltration of many plants and the simultaneous analysis of gene expression from several different genetic constructs on a single plant [34]. It is important to note that in cases of transient expression with *A. tumefaciens*, the transgene present in the T-DNA is transported to the nucleus and integrated into the plant genome in the usual way. However, its expression is transient because the infiltrated tissues rarely divide, and their viability is limited by the presence of agrobacteria in the intercellular space.

1.1.2 Stable Gene Expression

Stable gene expression refers to the expression of foreign genes in transformed plants over time and across generations [23]. During stable transformation, foreign genetic material enters plant cells, moves to the

nucleus, and integrates into the nuclear genome of a cell that can regenerate a whole plant, resulting in all offspring inheriting the foreign genetic material [23,35]. Currently, most transgenic plants are obtained by nuclear genome transformation using *A. tumefaciens*, as described above. Stable transformation has the advantage of producing many regenerated transgenic plants. However, this method is time consuming and not optimized for all plant species [23,35]. The expression levels of the transgenes vary between different transgenic lines due to random insertion of the foreign genetic material at different sites in the genome which might lead to gene silencing due to positional effects [36].

Clough and Bent developed a method for transforming plants with inflorescences containing a large number of flowers, such as *A. thaliana* [37]. The floral dip method for stable gene expression involves immersing plant inflorescences in a suspension of agrobacteria for a specific duration. During the floral immersion method, transformation with *A. tumefaciens* occurs primarily in the female gametophyte before fertilization [38]. As a result, the embryos resulting from the ovules transformed with T-DNA will be hemizygous after double fertilization, as they will contain only a single copy of the T-DNA, which is of maternal origin [37]. Subsequently, mature seeds are collected and germinated in a nutrient medium with a selective agent to identify the transformed plants. This method has also been applied to other plant species, such as *Brassica napus* [39], *Linum usitatissimum* [40], *Lepidium campestre* [41], and *Setaria viridis* [42], among others. The floral dip method has been used to obtain stable transformants of various *A. caudatus* cultivars, including Kremoviy ranni, Karmin, Helios, and Rushnychok, as well as the Sterk cultivar, which is a hybrid between *A. caudatus* and *A. paniculatus* [43,44]; *A. retroflexus*, *A. viridis*, and *A. cruentus* cv. Bagryanyi [45,46].

1.2 Cultivated Amaranth for Biotechnological Applications

1.2.1 Taxonomic and Morphological Description of Studied Cultivars

The *Amaranthus* genus belongs to the *Amaranthaceae* family, specifically the subfamily *Amaranthoideae*, tribe *Amarantheae*, and subtribe *Amaranthinae* [47]. However, due to the high morphological variability of the species, the presence of hybrids, and the lack of clear distinguishing characteristics, the taxonomic classification of the genus *Amaranthus* is complex. This complexity has led to misidentifications in nomenclature [48,49]. The classification of *Amaranthus* is typically based on inflorescence and flower morphology, as well as whether a species is monoecious or dioecious [50].

Amaranthus species are considered pseudocereals [48,51], because they produce many seeds that are consumed as grains. Species within the genus contain concentric rings of vascular bundles and efficiently fix carbon through a C4-type photosynthetic pathway. However, based on key their morphological characteristics, they are included in the class of dicotyledons [52]. *Amaranthus* are autogamous species that allow for controlled crossings and traditional genetic studies, as it produces up to 50,000 seeds per plant [53]. Additionally, amaranth has a short life cycle of about three months from germination to seed production. Its estimated genome size is 466 megabases, and a draft sequence has already been published [54].

The historical evidence suggests that ancient pre-Hispanic civilizations, such as Aztecs, Incas and Mayas cultivated and used several species of *Amaranthus* approximately 6,000–8,000 years ago [55]. One of this species *A. caudatus*, also referred to as 'kiwicha', is indigenous to the higher-altitude regions of Bolivia, Perú, and Ecuador [56]. *A. cruentus* is believed to have originated in Mexico and was utilized by the Mayans and Aztecs [55]. They considered it a high-yielding cultivated plant and appreciated its nutritional value.

However, its consumption was replaced by cereals during the colonization process after the arrival of the Spanish [55,57].

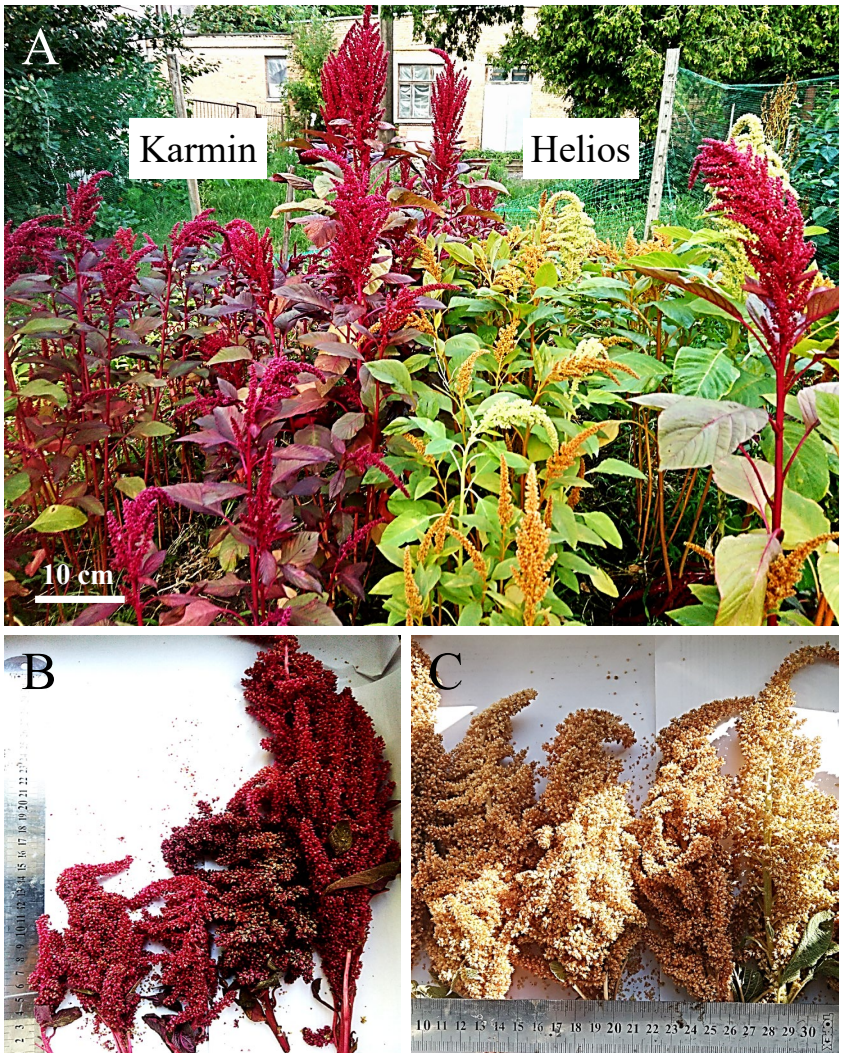

Figure 1. Morphology of *A. caudatus* cv. Karmin and Helios. (A) Overall plant growth and details of mature inflorescence (B, Karmin; C, Helios).

The Ukrainian varieties of *A. caudatus*, Karmin and Helios, are known for their high fiber content and early maturation [58–60]. These annual

herbaceous varieties grow up to 190–200 cm in height and have violet inflorescences. Their lateral branches grow at a 40-degree angle to the main stem, causing the inflorescence to spread laterally [58]. Karmin leaves are oval-shaped and violet-green, and the grain is white (Figure 1A, B) [58,60]. The Karmin variety was registered in 2000 [58, 60]. The Helios variety is an early-maturing grain variety that was registered in Ukraine in 2011 [58,60]. The height of Helios plants is 150–170 cm, and their inflorescences turn orange when mature (Figure 1B, C) [58,60].

1.2.2 Biochemical Composition and Usage of Amaranth-Based Products

Amaranth tissues are composed of proteins, fats, carbohydrates, minerals, and vitamins [61]. The plant's leaves, stems, and seeds have high protein content and can be used in food [62]. The protein fraction of amaranth is made up of albumins, globulins, gliadins, and glutens [63]. Their seeds contain 18 of the 22 known amino acids, making it the cultivated plant with the highest proportion of amino acids [63].

Amaranth species have a wide range of applications in various industries, including food, medicine, cosmetology, and textile. They are also used as a fodder plant in agriculture [63,64]. Due to their nutritional properties and adaptability, Amaranth species are emerging as a potential replacement for most common cereals, making them a promising crop for the new millennium [65]. The nutritional properties, together with the great adaptability of the species of this genus, position it as a new millennium crop [65]. Amaranth is used as an ingredient in various food products to increase their nutritional value and add flavor and color to dishes [63,64]. It is also used as a raw material in the pharmaceutical industry and non-traditional medicine, and as a component in anti-aging and sun protection creams in cosmetology. In the textile industry, amaranth serves as a natural color, providing an eco-friendly solution for coloring

materials [63]. In the textile industry amaranth serves as a natural color, providing eco-friendly solution for coloring materials [63]. In the agricultural sector, amaranth is utilized as a fodder plant for cattle and poultry, providing an inexpensive source of minerals, vitamins, and proteins [63].

1.2.3 Recent Advances in Amaranth Biotechnology

Effective protocols for in vitro regeneration of whole plants are essential for the application of various biotechnological tools, such as stable transgene expression using *Agrobacterium* spp. However, due to the high content of auxins in their tissues, most amaranth species have low regeneration capacity, which may suppress de novo shoot formation [66,67]. As a result, there are limited protocols available for the effective regeneration of *Amaranthus* species [68,69]. Due to the low regenerative capacity in amaranth species, it is necessary to search for alternative methods to deliver transgenes into plant tissues, such as vacuum infiltration or agroinjection [69].

In general, callus formation and regeneration in plants are influenced by various factors, including the physiological characteristics of the species, plant varieties, the ratio of endogenous hormones, and the type and age of the explants [68–71]. When designing experiments for amaranth regeneration, it is important to consider all these factors. Callus induction can be achieved in almost all amaranth species. The most used *Amaranthus* species for transformation are *A. caudatus*, *A. tricolor*, *A. cruentus*, and *A. hypochondriacus* [69]. Regenerated plants have been obtained after agrobacterial transformation using species mentioned in the table (Table 1). *Agrobacterium*-mediated stable transformation is the most used method for transforming amaranth species. In most experiments, the *A. rhizogenes* A4 strain [44,68,69,72] was found to be more effective, while the use of *A. tumefaciens* strains BGV [68] and GV3101 [43] was less successful [69].

Table 1. Achievements in callus induction and regeneration of *Amaranthus* species.

Species	The genes which were incorporated after transformation	References	Purpose	References
A. caudatus	*rolB, uidA, bar, GFP, INFa2b, nptII*	[43,44,72–74]	Callus induction Microclonal multiplication Regeneration	[75–77] [77] [76]
A. cruentus	*hph, uidA, ARGOS-like, rolB*	[46,78–80]	Callus induction and regeneration Callus induction Callus induction and regeneration	[46,66,67,76,79,81–83] [84,85] [69,70,82,83]
A. hypohondriacus	*uidA, nptII, GFP, bar, rolB*	[69,70,86]	Callus induction Microclonal multiplication Regeneration	[85,87] [87] [76,81]
A. hybridus	*GFP, bar, rolB*	[69]	Callus induction and regeneration Callus induction Regeneration	[66–69] [81] [76]
A. retroflexus	*hph, ARGOS-like*	[45,46]	Not conducted	
A. spinosus	*rolB*	[88]	Callus induction regeneration	[89] [90]
A. tricolor	*rolB, nptII, uidA, gmIFS, GFP*	[71,90–93]	Callus induction and regeneration Callus induction Regeneration	[83] [89] [71,91]
A. trisis	*uidA*	[94]	Not conducted	
*A. tubeculatus**	*uidA, hph*	[95]	Callus induction and regeneration	[95]
A. viridis	*hph*	[46]	Not conducted	
A. edulis	Not conducted		Regeneration	[96]
A. gargenticus	Not conducted		Callus induction	[97]
A. paniculatus	Not conducted		Callus induction and regeneration	[98]

1.3 Cultivated Tomato for Biotechnological Applications

1.3.1 Taxonomic and Morphological Description of Studied Cultivars

The cultivated tomato (*Solanum lycopersicum*) is a dicotyledonous vascular plant belonging to the nightshade family (*Solanaceae*), which comprises over 3,000 species, distributed across 90 genera [99]. Other important species in this family include potatoes (*Solanum tuberosum*), eggplants (*Solanum melongena*), medicinal plants like nightshade (*Atropa belladona*), and ornamentals such as petunias (*Petunia × hybrida*) [99,100]. The genus *Solanum* encompasses approximately 1,500 species [99]. Within this genus, the tomato clade (section *Lycopersicon*) consists of the cultivated tomato and 12 wild relatives, all of which are indigenous to western South America [101]. Several hypotheses have been proposed regarding the origin, domestication, and dispersal of the tomato. These hypotheses are based on data obtained through single nucleotide analysis or whole genome sequencing of a wide range of wild tomato species and *S. lycopersicum* var. *lycopersicum* genotypes [102,103]. According to the theories of Blanca and Razifard, *Solanum pimpinellifolium*, a species of wild tomato native to Perú and Ecuador, is the ancestor of the cultivated tomato, *S. lycopersicum* [104]. *S. pimpinellifolium* was dispersed from Perú and Ecuador and into Mesoamérica through human action . This led to its semi-domestication and the creation of an intermediate form of domesticated tomato known as *S. lycopersicum* var. *cerasiforme*, which later returned to its area of origin in Perú and Ecuador. In a subsequent wave of migration, the new tomato varieties spread to México, where a second, more successful domestication was carried out, leading to the appearance of *S. lycopersicum* var. *lycopersicum*. Hernán Cortés introduced these cultivated tomatoes to Spain in 1512 from the Aztec city of Tenochtitlán [105]. Similar tomatoes are thought to have spread throughout Europe from Spain [105,106].

Tomato is a valuable model plant system due to its short life cycle, ease of cultivation in diverse environmental conditions, and ability to reproduce through autogamy with controlled pollination and hybridization [107]. Furthermore, tomato has a relatively small genome, approximately 950 megabases, with only 12 pairs of chromosomes that have been sequenced [108,109]. Several genetic tools are available for studying tomatoes, including collections of mutants, genetic markers, linkage maps, and many characterized varieties [107].

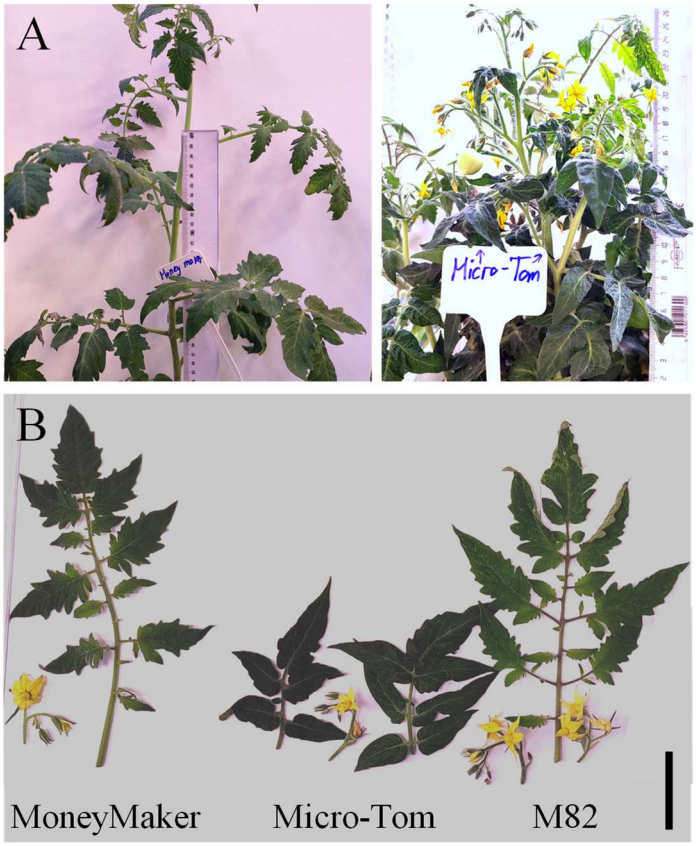

Figure 2. Morphology of studied *S. lycopersicum* cultivars. (A) Overall plant growth and (B) details of leaves and flowers. Scale bar: 4 mm.

S. lycopersicum var. Micro-Tom is a cultivar of tomato that is commonly used as a model for genetic studies in Solanaceae due to its small size (15-30 cm) and short life cycle (12 weeks). It was created by crossing the 'Florida basket' and 'Ohio 4013-3' varieties [110,111]. The plant has a compact structure with dark green leaves and a reduced number and size of the internodes. The leaves of Micro-Tom have a rough surface with downward-curled margins. The phenotypic characteristics of Micro-Tom are determined by specific mutations: *dwarf* (*d*), *enhancer of jointless* (*ej-2*^{w}), *Immunity to fusarium wilt* (*I*), *miniature* (*mnt*), *self-pruning* (*sp*), *uniform ripening* (*u*), and *Stemphylium resistance* (*Sm*) [110]. The small size of Micro-Tom plants is associated with the mutations *d* and *mnt* [112]. The *d* gene encodes a cytochrome P450 enzyme associated with the synthesis of brassinosteroids. *Mnt* is associated with the gibberellin pathway [110]. Mutations in the *SP* gene cause the determinate growth habit [113]. Mutations of the *U* gene correspond to the uniform light green color of unripe fruits [114].

M82 is a commercial *S. lycopersicum* cultivar obtained in 1994, with the genetic configuration *sp*; *u*; *obscuravenosa* (*obv*); *I*; *Verticillium resistance* (*Ve*) [115]. The plant is tall, can reach approximately 1 m and the cultivar has a long-life cycle (90–110 days from germination until fruit maturation) [116,117]. The *obv* mutation causes leaf veins to appear green, due to presence of chloroplasts in epidermal layer beneath veins [112]. M82 are resistant to *Fusarium oxysporum*, as determined by the *I* mutation, and to *Verticillium* spp. because of mutations in the *Ve* gene [112]. *S. lycopersicum* var Moneymaker is a popular tomato cultivar which dates to 1913 [118], with a known genetic configuration: *sp*$^{+}$; *ej-2*^{w}; *u*; *obv*$^{+}$. Mature plants can reach up to 2 meters high. The plants have increased inflorescence subdivision, what is caused by mutation in *ej-2*^{w} [119]. More details of the studied tomato genotypes are shown in Figure 2 and Table 2 (see page 19).

1.3.2 Health-Promoting Components of Tomato Fruits

Tomato is an important agricultural plant worldwide. In recent years, the consumption of tomatoes has increased, as tomatoes are supplied to the market fresh and processed forms. According to the year report of Food and Agriculture Organization, the total world production of tomatoes in 2021 for both processing and fresh consumption reached 189.1 million metric tons in 2021 [120]. China being the leading producer with a total of 67.5 million metric tons (36% of world production) [120]. Spain produced 4.7 million metric tons, ranking seven in the world for tomato production [120]. Tomato fruits are an important source of bioactive compounds such as vitamins, antioxidants, and substances with anti-tumor properties [121]. Compounds with antioxidant properties include carotenoids, phenolic compounds, and phenolic acids [122]. They provide effective protection by neutralizing free radicals, which are associated with the development of several degenerative diseases [123]. The consumption of tomatoes can have a positive effect on the reduction of cholesterol levels in blood [124,125].

Tomato fruits are rich in carotenoids, such as lycopene, β-carotene, and lutein [125], which may control oxidative stress and inflammation [122], suppress the development of tumors [126,127]. In addition, β-carotene has the function of preventing of photo-oxidative damage [128] and myocardial infarction, as well as inhibits the development of atherosclerosis [129]. Tomato fruits contain tocopherol, which inhibits lipid peroxidation, suppresses the development of cardiovascular diseases [130,131], and reduces the risk of type 2 diabetes [132]. Additionally, tomato fruits contain phenolic compounds, such as flavonoids, phenolic acids, and tannins [132]. Flavonoids have anti-inflammatory activity in the colon [133], inhibit inflammation [134], and reduce the risk of gastric cancer [135–137]. Phenolic acids have been shown to protect against DNA oxidation and exhibit antitumor activity [138–140]. Tannins, on the other hand, have been found

to suppress adipogenesis and possess antibacterial, antiviral [141], and anticarcinogenic [142] properties. Hence, regular consumption of tomatoes may reduce the risk of inflammation, cancer, and chronic diseases such as atherosclerosis, cardiovascular disease, hypertension, diabetes, and obesity.

1.3.3 Recent Advances in Tomato Biotechnology

The initial study on genetic transformation of tomatoes through *Agrobacterium* spp. was published in 1986 [143]. Since then, efficient transformation protocols have been established for various tomato varieties using different tissue explants, including cotyledons, hypocotyls, leaves, flower buds, and even fruits [144–149]. However, successful regeneration and transformation results have only been obtained for a limited number of commercial varieties. Regeneration efficiency from some genotypes, such as Micro-Tom or Moneymaker, is still suboptimal.

Several methods have been successfully used for tomato transformation including stable transformation mediated by co-cultivation with *A. tumefaciens* [150] and *A. rhizogenes* [151], agroinfiltration [145,147], agroinjection of leaves [152,153] and fruits [145], and floral-dip transformation [144]. However, the main method of tomato transformation remains *Agrobacterium*-mediated stable transformation. Other methods are typically used to overcome possible problems related to whole plant regeneration after transforming explants [107]. Agroinfiltration and virus induced gene silencing are alternative method which can be used for functional analysis of transgenes [145,146]. The floral-dip method, commonly used in *A. thaliana* [37], has also been employed for tomato transformation with an efficiency of 12% to 23% [132]. The most common methods for direct transferring genetic material are DNA microinjection [154], electroporation [155], polyethylene glycol gene transfer [156], and biolistics [157], which have been widely used for successful tomato transformation.

1.4 Objectives of my Doctoral Thesis

The impact of climate change and biological stresses caused by human activities on agriculture has resulted in significant losses. To address this issue, new biotechnological tools are required for emerging and widely used agricultural species. The objective is to develop these tools and mitigate the negative effects on crops. The two scientific articles included in this report are framed in this context and constitute the Doctoral Thesis, aimed at:

- Optimize the conditions for callus induction and shoot regeneration from tomato explants.
- Characterize the systems used for gene editing in tomato and related species.

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2. OVERALL SUMMARY OF MATERIALS AND METHODS

2.1 Plant Material

The plant material used in this study consisted of two local Ukrainian cultivars of *A. caudatus*, three genotypes of *S. lycopersicum* var. *cerasiforme*, and three genotypes of S. *lycopersicum* var. *lycopersicum* (Table 2). The *A. caudatus* seeds were obtained from the M. M. Hryshko National Botanical Garden (Kyiv, Ukraine). Tomato seeds were obtained from the seed bank of Instituto Universitario de Conservación y Mejora de la Agrodiversidad Valenciana (Universitat Politècnica de València, Spain). The bacterial vectors used in this work (Table 3) were provided by Nomad Bioscience GmbH (Haale, Germany), and the bacterial strains were obtained from the Institute of Cell Biology and Genetic Engineering NAS (Kyiv, Ukraine).

Table 2. Plant material used in this work.

Plant species	Accession[1]	Cultivar name	Collection site
A. caudatus	09403001	Helios	Ukraine
A. caudatus	98099002	Karmin	Ukraine
S. lycopersicum var. *lycopersicum*	LA3475	M82	
S. lycopersicum var. *lycopersicum*	LA3911	Micro-Tom	
S. lycopersicum var. *lycopersicum*	LA2706	Moneymaker	
S. lycopersicum var. *cerasiforme*	BGV 007910		México
S. lycopersicum var. *cerasiforme*	BGV 007927		México
S. lycopersicum var. *cerasiforme*	BGV 016054		Perú

[1]Accession numbers for tomato genotypes were obtained from the COMAV germplasm bank (BGV…) [158] and the C.M. Rick Tomato Genetics Resource Center database (LA…) [159]. For Amaranth genotypes, the codes correspond to register of varieties recommended for growing in Ukraine [59]

Table 3. Bacterial strains used in this work.

Bacterial strains	Vector	Antibiotics for bacterial selection	Reporter gene	Plant selection gene
A. tumefaciens GV3101	pICBV19	50 mg/L rifampicin, 25 mg/L gentamicin	*uidA*	*bar*
A. tumefaciens GV3101	pNMD2501	100 mg/L ampicillin, 25 mg/L gentamicin	*GFP*	

Information about vectors is available in publications [44,160], and [161].

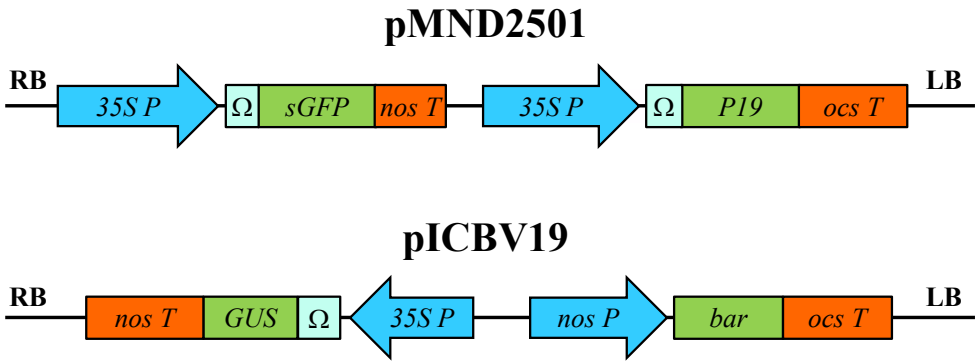

Figure 3. Schematic representation of the T-DNA region of the pICBV19 and pMND2501 vectors. *nos P* and *nos T* promoter and terminator sequences of the *A. tumefaciens nopaline synthase* gene, respectively; *ocs T*, terminator sequence of the *A. tumefaciens octopine synthase gene*; Ω, 5' omega leader sequence of tobacco mosaic virus used as a translational enhancer; *sGFP*, the *GFP* reporter gene; *P19*, the gene encoding the P19 protein of tomato bushy stunt virus, which is used as a suppressor of post transcriptional gene silencing; *uidA*, the *uidA* reporter gene sequence from *E. coli* encoding the β-glucuronidase A enzyme; *bar*, the *bar* gene sequence from *Streptomyces hygroscopicus*, encoding the phosphinothricin N-acetyltransferase enzyme; *LB* and *RB*, left and right borders of *A. tumefaciens* T-DNA, respectively. Schemes have been redrawn from [44] (pICBV19) and [161] (pMND2501).

2.2 Establishment of Transient Expression of Genes in *A. caudatus* through vacuum infiltration

Seedlings of *A. caudatus* cv. Karmin and cv. Helios at different ages, including one-day-old, ten-day-old, and two-month-old plants, were used for transformation. Two strains of *A. tumefaciens* GV3101 were used, one carrying the pICBV19 vector [44,160] and the other carrying the pMND2501 vector [161]. The *uidA* and *GFP* genes in both constructs were driven by the cauliflower mosaic virus (CaMV) 35S promoter (Figure 3). The bacterial strains were grown in liquid Luria-Bertani medium with appropriate antibiotic concentrations for 24 hours. Afterwards, the liquid cultures were centrifuged at 20°C (4500 revolutions per minute, 20 minutes) and the bacterial pellet was resuspended in a 10 mM $MgSO_4$ solution.

The study involved the vacuum infiltration of one-day-old (*n*=100), ten-day-old (*n*=100), and two-month-old (*n*=30) plants of each variety with resuspended *A. tumefaciens* cultures under a pressure of 0.1 MPa for 5–10 minutes at a temperature of 22–24°C.

On the fifth day after the infiltration, the detection of the β-glucuronidase activity encoded by the *uidA* gene of *E. coli* was performed in the leaves of adult plants and whole young seedlings using the histochemical assay in the presence of 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-gluc) substrate. The GFP was detected in the young seedlings after infiltration through visual evaluation of the green fluorescence in the infiltrated tissue. The results were subsequently verified through statistical analysis, as detailed in the Materials and Methods section of the second article on pages 70-71.

2.3 Optimization of Callus Induction and Shoot Regeneration from Tomato Cotyledon Explants

Seeds were germinated and cultivated following the procedure outlined in the Materials and Methods section of the first article on pages 65

and 66. Five different experiments (E1 to E5) were conducted. For experiments E1, E2, and E3, 7-day-old cotyledon explants were cut into two parts (0.51±0.18 cm^2 each) and placed in 6-well plates with Murashige and Skoog basal salt medium [162]. Various media variants containing 2% sucrose and 0.8% agar supplemented with zeatin alone (1, 2, 3 mg/L) or in combination with indole acetic acid (0, 0.1 and 0.5 mg/L) were used in these experiments. For the E4 experiment, cotyledons obtained from 7-day-old seedlings were cut into three parts (apical, central, and basal, each with a size of 0.27±0.17 cm^2) and transferred to Murashige and Skoog medium with 2% sucrose, 0.8% agar, 1 or 2 mg/L zeatin, and 0.5 mg/L indole acetic acid.

Explants were cultivated under the following conditions: a 16-hour light emitting diode and 8-hour dark period at a temperature of 23–24°C. The experiments were conducted in three biological replications with 15 explants per genotype and treatment (experiments E1, E2 and E3; *n*=585 explants) or with 12 explants per each genotype and treatment (experiment E4; *n* = 432 explants). The plates were incubated for up to 1.5 months and scanned weekly. During this period, explants were transferred every two weeks to fresh media, supplemented with the same combination and concentration of growth stimulants. The presence of callus and shoot primordia was visually identified daily using a stereomicroscope. The explant and callus areas were quantified separately from scanned images using ImageJ software after freehand line drawing. The results were analyzed using appropriate statistical methods, as described in the Materials and Methods section of the first article on pages 65 and 66.

2.4 Characteristics of Gene Editing Systems Used in Cultivated Tomato and Other Related Species

To characterize the editing systems used in tomato and related species, we conducted a comprehensive analysis of the available literature (see the

second article on pages 75 to 96). Our objective was to systematize experimental data from other authors on these species. We methodically searched three databases (PubMed, Web of Science, and Scopus) using the keywords 'Cas9' and 'tomato' with time restrictions from January 1, 2014, to December 31, 2023.

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3. DISCUSSION

3.1 Transient Expression of *uidA* and *GFP* Genes in *A. caudatus* through Vacuum Infiltration

Cultivated *Amaranthus* spp. plants have high agronomic potential because of their balanced composition of amino acids and other components, resulting in high nutritional value. These plants are an ideal platform for producing various recombinant proteins due to their complex metabolism. However, the low regenerative capacity observed in most amaranth species requires the use of specific transformation methods to overcome this critical step for stable plant transformation. Transformation protocols are typically standardized for model plant species, such as *Arabidopsis thaliana* [163] and *Nicotiana benthamiana* [164]. Establishing a protocol for recalcitrant plant species can be particularly challenging [165].

Using the floral-dip transformation protocol available for *Amaranthus* spp., we obtained stable transformants of *A. caudatus* with a very low transformation efficiency (0.1%–2.2%). Other authors have reported varying ranges of transformation efficiencies in diverse amaranth species, with most studies falling between 1.3% [86] and 24.2% [90]. Only one study reported a higher efficiency of 50.0% to 66.8% [68]. Infiltration with agrobacteria is a viable alternative to stable transformation for achieving transient expression of transgenes. This method is simple, requires minimal equipment and plant material, and produces results quickly. It also allows for the analysis of multiple constructs in parallel and in different genetic backgrounds. Experiments on various plant species have demonstrated that vacuum infiltration of leaves or young seedlings with *Agrobacterium tumefaciens* is an effective method for achieving transient gene expression in recalcitrant plant species. This technique has been successfully applied to *Theobroma cacao* [165] and several citrus species [166]. The vacuum infiltration method can also be useful for other procedures involving DNA transfer to plant cells, such

as virus-induced genetic silencing, RNA interference, or CRISPR/Cas genome editing [165].

We applied a vacuum infiltration method to young amaranth seedlings of different ages, from one-day-old seedlings to two-months-old plants, to transiently express *uidA* or *GFP* transgene under the control of the constitutive promoter CaMV 35S. The percentage of vacuum-infiltrated plants that tested positive for the histochemical reaction driven by the *uidA* transgene decreased as the seedlings aged and was dependent on the cultivar. In one-day-old seedlings of both Helios and Karmin cultivars, most plant tissues, including roots, hypocotyl, and cotyledons were positively stained for the β–glucuronidase reaction with high efficiencies (95% and 93%, respectively). In Helios, the percentage of these was reduced to 61.3% in ten-day-old seedlings and 16.0% in two-month-old plants. In Karmin, the percentage was reduced to 41.6% in ten-day-old seedlings and 12.0% in two-month-old plants. The variation in expression may be caused by differences in cultivar genotypes, which could affect their susceptibility to *A. tumefaciens* infection. UV microscopy was used to visually analyze GFP expression in amaranth tissues. The results of *GFP* gene expression were comparable to those obtained for the *uidA* gene. Strong expression of the *GFP* gene, driven by the CaMV 35S promoter, was observed in transgenic tissues and hairy roots of transformed *Amaranthus* spp. [68].

It is worth noting that there is only one other report of transient gene expression in *A. tricolor*, which was obtained using the agroinjection method [93]. In our work, the expression of *uidA* and *GFP* was transient and mainly observed in the vascular bundles and midrib of leaves in ten-day-old seedlings. In two-month-old plants, the expression of these genes was limited to the vascular bundles and midrib of leaves, confirming the findings of previous studies [167]. The use of very young seedlings (4 days or less) allows for rapid analysis of results with minimal manipulation. Our results on the

localization of *uidA* gene expression in plant tissues and organs during transient expression are consistent with previous studies [167]. In experiments involving vacuum or syringe infiltration of *Catharanthus roseus* seedlings using the *uidA* gene driven by the CaMV 35S promoter, the youngest seedlings exhibited the highest transformation efficiencies with vacuum infiltration [168]. Similar results were reported for Arabidopsis, tobacco, and tomato, where the transient expression of the *GFP* gene was driven by the CaMV 35S promoter in cotyledon leaves of very young seedlings [163]. Typically, transgene expression levels are high in young vacuum-infiltrated seedlings, enabling biochemical assays with transgenic proteins, such as Western blotting [163].

In summary, the vacuum infiltration method that we developed enables testing of various new constructs in seedlings of amaranth with diverse genetic backgrounds. This method is species-independent, which is a significant advantage compared to tissue culture-based methods. It can be used for biochemical and immunoblotting analyses, promoter testing, protein localization, and protein-protein interaction studies. In addition, we demonstrate the versatility of our method by successfully applying it to *Helianthus annus* and *Physalis peruviana* [169,170]. Moreover, it can be used for the transformation of mutant and transgenic plant lines carrying lethal genes that do not develop until the true leaf stage [163]. Our method is also useful for construct screening and characterization before conducting stable transformation in *Amaranthus* spp.

3.2 Optimization of Callus Induction and Shoot Regeneration from Tomato Cotyledon Explants

Improved procedures for obtaining regenerated shoots are always desirable because they reduce labor and resource costs and allow for fast organ formation and early evaluation of obtained transgenic lines. In the third paper, we evaluated various regeneration methods in different tomato genotypes, including commercial cultivars and ancient accessions. We evaluated the effect of different hormones on the regenerative capacity of tomato cotyledons and assessed the regenerative capacity in different parts of the cotyledons. Additionally, we conducted a cellular study of this process (see the first article on pages 55 to 73).

Our results indicate that the addition of exogenous auxins to the growth medium significantly enhances the effect of zeatin on the induction of callus and shoot regeneration. The addition of 3.0 mg/L of zeatin alone had a negative effect on callus formation in Micro-Tom. The time required for callus formation ranged from approximately 15 to 19 days. 2,4-dichlorophenoxyacetic acid was not used due to its potential to cause physiological changes or somaclonal variations [171]. The next step in our work was to investigate the influence of growth stimulants influence on shoot regeneration. According to our results, shoot regeneration of M82 explants was dependent on the increasing levels of exogenously applied indole-acetic acid. The formation of shoot apical meristem primordia was strongly dependent on genotype, with Moneymaker producing the highest number of them. The lower regenerative capacity of Micro-Tom compared to M82 may be caused by Micro-Tom's genetic background, due to mutations in genes related to gibberellin signaling and brassinosteroid biosynthesis [110].

Previous studies have confirmed that the addition of growth stimulants to a medium plays a crucial role in regulating organ formation, callus induction, and rooting [172]. It was shown that adding 0.05 mg/L or 0.1 mg/L

indole-acetic acid to a medium containing zeatin reduced the time required for shoot regeneration in M82 [150]. Indirect plant regeneration was achieved for seven-day-old cotyledon explants of Rio Grande, Roma, and hybrid 17905 × M82 varieties on Murashige and Skoog basal salt media supplemented with various combinations and concentrations of growth regulators [173]. Multiple shoots were obtained from callus after three weeks of cultivation on medium supplemented with 3 mg/L of 6-benzylaminopurine and 0.1 mg/L of indole-acetic acid. Additionally, it was observed that the number of shoots from cotyledon explants of Arka Vikas and Pusa Early Dwarf increased when 2.0 mg/L of zeatin was added in combination with 0.1 mg/L of indole-acetic acid [174]. Positive effect on tomato shoot regeneration of zeatin in combination with indole-acetic acid was confirmed in range of publications [107]. Kantor et al. reported that the highest rates of regeneration were achieved using Murashige and Skoog medium with the addition of 1 mg/L of zeatin and 0.05 mg/L of indole-acetic acid resulted in the highest rates of regeneration [175]. Zhang found that the most effective medium for shoot regeneration from cotyledon explants was Murashige and Skoog supplemented with 2 mg/L of zeatin and 0.01 mg/L of indole-acetic acid was the most effective for shoot regeneration from cotyledon explant [176]. The influence of growth stimulant combinations and concentrations on shoot regeneration was also confirmed in experiments with the Micro-Tom cultivar [177].

The type of explant may also affect regeneration rates is [176]. Therefore, we evaluated the influence of the basal, central, and distal regions of the cotyledon on callogenesis and shoot regeneration of six tomato genotypes (see the first article on pages 55 to 73). Calli formation and growth occurred at both cut ends (proximal and distal) of each explant shortly after excision in all cases. Regardless of the cotyledon region used, the proximal region of the explants exhibited more intense callus growth. Shoot

regeneration rates varied among the six genotypes studied and were dependent on the region and genotype. The basal cotyledon region was found to be more effective for regeneration than the apical region. The minimum explant size required for effective regeneration under our conditions was approximately 0.20 cm^2. The size of the explant influences the tissue's response to growth stimulants. Smaller explants are more difficult to cultivate [178]. Larger explants likely contain more nutrients and plant growth regulators to support their autonomous growth after excision [178]. Previous studies have shown that tobacco explants taken from different parts of the stem (base, middle, or top) have varying morphogenetic potential [179]. Due to varying endogenous rates of hormones in different parts of the same plant, explants from different origins may have different levels of growth regulators [179]. As a result, differences in hormone balance in the organs or tissue can lead to variable in vitro responses [179].

Genotype of tomato can cause strong influence on tomato shoot induction [180]. Most genotypes produced more shoot apical meristem primordia from the basal region than from the apical and central regions. In our experiments Micro-Tom and genotypes BGV 007910 and BGV 007927 had a low rate of adventitious shoot formation. In a previous study, the shoot regenerative capacity of hypocotyl and leaf discs of Moneymaker explants was evaluated [181]. They induced adventitious shoot formation using a combination of zeatin and indole acetic acid [182]. The optimal conditions for shoot regeneration from Micro-Tom cotyledon leaf explants were evaluated (see the first article on pages 55 to 73). We found that Murashige and Skoog basal salt medium supplemented with 1 mg/L zeatin, 0.1 mg/L indole-acetic acid, and 3% sucrose was the most effective. Non-significant differences in shoot regeneration were detected with respect to explant age up to 10 days. Our results for several tomato genotypes showed that de novo shoot formation in Micro-Tom was higher in the basal cotyledons than in the central

or apical explants. A greater positive correlation was observed when the abaxial side of the explant touched the medium [173]. In summary, our findings suggest that the best conditions for de novo shoot formation in tomato involve using the basal segment of young cotyledons (seven days old) placed in a culture medium (Murashige and Skoog basal salts, 1 mg/L zeatin, 0.1 mg/L indole-acetic-acid, and 2% sucrose) on their abaxial surface. It is important to note that the calli formed in tomato explants in response to treatment with zeatin and indole-acetic-acid had a heterogeneous nature. Two types of calli were visually detected and distinguished: soft or friable calli, which were more often formed at the edges of the explants and near the cut region, and dense calli. Our procedure resulted in a higher number of shoots per explant compared to previous studies [180]. Additionally, our analysis expands the understanding of the regenerative capacity of cotyledon explants by including Moneymaker, Micro-Tom, M82, and three ancient tomato cultivars (see the first article on pages 55 to 73). It was determined that the optimal concentrations of growth stimulants are 1 mg/L of zeatin and 0.5 mg/L of indole-acetic acid. Additionally, the cotyledon regions with the highest regeneration capacity were identified. The presented method offers several advantages. Firstly, it can be performed in six-well plates, allowing for high throughput escalation. Secondly, it enables rapid regeneration. Finally, it can be applied to a wide range of tomato genotypes. This method is useful for performing plant transformation and gene editing experiments in tomato.

3.3 Characteristics of Gene Editing Systems Used in Cultivated Tomato and Other Related Species

Gene editing tools such as zinc finger nucleases, transcription activator-like effector nucleases, and clustered regularly interspaced short palindromic repeats/CRISPR-associated (CRISPR/Cas) have been used in tomato for 15 years (see the second article on pages 75 to 96). The advances in crop gene editing techniques, particularly the CRISPR/Cas system, have revolutionized

the field of gene modification by providing efficient and precise tools. This technique has the potential to enhance crop traits, improve yield, increase tolerance to biotic and abiotic stresses, and develop cultivars with desirable traits.

The CRISPR/Cas system has been shown to be effective in modifying multiple genes in cultivated tomato varieties and related wild species, indicating its potential for crop improvement. Cas9 was the most used among the different Cas proteins for mutagenesis of tomatoes. More than ten tomato cultivars were edited in a short period of time. Successful delivery of editing tools was achieved through different methods. Agrobacterium-mediated delivery was the most used method for both *S. lycopersicum* and wild tomato species. However, the agroinfiltration method was more effective in achieving higher editing rates. Recent literature suggests that editing genes related to the quantitative and qualitative characteristics of tomato fruits, as well as genes that improve resistance to biotic stresses, is highly desirable. Therefore, the use of the CRISPR/Cas will accelerate the development of tomato cultivars that are tolerant to biotic and abiotic stresses. In addition, CRISPR/Cas editing can be used to modify essential genes in tomato plants, resulting in new varieties or the domestication of heirloom species. This can lead to improved fruit qualities and architecture, as well as increased levels of vitamins and other beneficial compounds for health.

Although gene editing has already yielded significant results for many wild and cultivated tomato species, the search for new types of Cas proteins or improvements to existing ones with flexible protospacer adjacent motif requirements and high on-target efficiency and low off-target efficiency continues. For instance, several new small orthologs of the Cas12 protein have been discovered and successfully used for representatives of the Solanaceae family [183]. Also, nickase variants of Cas9 and Cas12, can be used

for genome editing purposes due to their ability to produce more predictable mutations at on-target sites and have lower off-target effects compared to Cas9. The discovery of new genome editing systems, such as TnpB and Fanzor [184,185], expands the range of available genome editing tools. However, their use in eukaryotes, particularly in plants, is still unknown [184].

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4. CONCLUSIONS AND FUTURE PROJECTION

4.1 Conclusions / *Conclusiones*

- An efficient method for callus formation and tomato regeneration was established / *Hemos establecido un método eficaz para la formación de callos y la regeneración en explantes de tomate*.
- The formation of callus in both M82 and Micro-Tom cultivars was influenced by the levels of indole-acetic acid and zeatin. When grown on a medium supplemented with 1 mg/L of zeatin and 0.5 mg/L of indole-acetic acid, Micro-Tom had a callus formation rate of 93.3%, while M82 had a callus formation rate of 92.0 % on a medium supplemented with 3 mg/L of zeatin and 0.5mg/L of indole-acetic acid / *Los niveles exógenos de ácido indolacético y zeatina influyeron en la formación de callos en los cultivares M82 y Micro-Tom. En un medio suplementado con 1 mg/L de zeatina y 0,5 mg/L de ácido indol-acético, Micro-Tom tuvo una tasa de formación de callo del 93,3%, mientras que M82 tuvo una tasa de formación de callo del 92,0% en un medio suplementado con 3 mg/L de zeatina y 0,5mg/L de ácido indol-acético.*
- The percentage of callus formation was higher in the basal region of the explants compared to the apical and central regions of the explants. Calli formed from the basal part of the explants tended to be larger. Among the tested genotypes, Moneymaker had the highest percentage of callus formation at 95.0%, while BGV 007927 had the lowest percentages of callus formation at 50.0% / *El porcentaje de formación de callos fue mayor en la región basal de los explantes que en las regiones apical y central de los mismos. Los callos formados a partir de la región basal de los explantes eran más grandes. Entre los genotipos ensayados, Money-maker tuvo el porcentaje más alto de formación de callos con un 95,0%, mientras que BGV 007927 tuvo los porcentajes más bajos con un 50,0%.*

- The formation of tomato shoots from calli was dependent on genotype and zeatin levels. The formation of the shoot primordia was negatively correlated with increasing zeatin levels in M82 and Micro-Tom cultivars. M82 exhibited a significantly higher proportion of explants with shoot primordia than Micro-Tom on a medium supplemented with 2 mg/L of zeatin and 0.5 mg/L of indole-acetic acid (89.9% and 34.5%, respectively) / *La formación de brotes de tomate a partir de callos dependió del geno-tipo y de los niveles exógenos de zeatina. La formación de brotes se corre-lacionó negativamente con el aumento en los niveles de zeatina en am-bos cultivares. M82 mostró una proporción significativamente mayor de explantes con brotes que Micro-Tom en un medio suplementado con 2 mg/L de zeatina y 0,5 mg/L de ácido indol-acético (89,9% y 34,5%, respec-tivamente).*
- For most genotypes, the number of shoot primordia was higher on the basal region than on the central and apical regions of the explants. M82 and Moneymaker, had the highest number of primordia, while BGV 007910 and Micro-Tom had the lowest / *En la mayoría de los genotipos estudiados, el número de brotes fue mayor en la región basal que en las regiones central y apical de los explantes. M82 y Moneymaker, tuvieron el mayor número de primordios, mientras que BGV 007910 y Micro-Tom tu-vieron el menor.*
- Our findings suggest that the optimal hormonal combination for callus growth in M82 and Micro-Tom cotyledon explants is 1 mg/L of zeatin and 0.1 mg/L of indole-acetic acid. Additionally, the best hormonal combina-tion for inducing higher shoot formation is 1.0 mg/L of zeatin and 0.5 mg/L of indole-acetic acid / *Nuestros resultados sugieren que la combi-nación hormonal óptima para el crecimiento de callo en los explantes de cotiledón de M82 y Micro-Tom es 1 mg/L de zeatina y 0,1 mg/L de ácido indol-acético. Además, la mejor combinación hormonal para inducir una*

mayor formación de brotes es 1,0 mg/L de zeatina y 0,5 mg/L de ácido indol-acético.

- We conducted a thorough analysis of tomato gene editing systems and found that the most used cultivars for editing are Micro-Tom, Moneymaker, M82, and Ailsa Craig. The CRISPR/Cas9 system is the most popular editing system. The most used method for delivering editing tools is *Agrobacterium tumefaciens*-mediated stable transformation. However, more effective gene editing can be achieved through the agroinfiltration method / *Realizamos un análisis exhaustivo de los sistemas de edición gené-tica del tomate y descubrimos que los cultivares más utilizados para la edi-ción son Micro-Tom, Moneymaker, M82 y Ailsa Craig. El sistema CRISPR/Cas9 es el sistema de edición más popular. El método más utilizado para suministrar herramientas de edición es la transformación estable me-diada por* Agrobacterium tumefaciens*. Sin embargo, el método de agroin-filtración es más eficaz para editar genes.*

4.2 Future Projection

The experiments conducted on *Amaranthus* spp. and tomatoes yielded promising results. We developed a method for transforming *A. caudatus* Helios and Karmin cultivars to achieve temporary gene expression. This is the first time that the expression of reporter genes after agroinfiltration in *A. caudatus* cultivars has been confirmed. The proposed method is simple and fast, with results obtainable just four days after the experiment begins. The Institute of Cell Biology and Genetic Engineering in Ukraine used the same method to test several other genetic vectors to confirm their functionality before attempting stable transformation. Additionally, this method allows for the confirmation of the initial susceptibility of experimental plants to specific bacterial strains. In the future, this transformation method can be used to obtain target proteins with medicinal properties from amaranth.

Furthermore, this method can efficiently deliver vectors with editing tools to produce mutants with downregulated or upregulated genes. This can enhance our comprehension of multiple gene pathways.

Our experiments on regeneration with *S. lycopersicum* cultivars showed that different genotypes respond differently to identical concentrations of growth stimulants. We developed an optimized regeneration methodology that was highly effective for all tested tomato plants. Our procedure allowed us to obtain initial results quickly. This methodology can be used as an intermediate step in stable *Agrobacterium*-mediated transformation experiments. The regeneration protocol I have developed is being currently used at the host laboratory for rapid testing of vector functionality with incorporated reporter genes and gene editing tools (*i.e.*, CRISPR/Cas). The proposed method will be used to obtain edited tomato mutants at the host laboratory.

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6. ANNEX I: Publications included as part of the Thesis

- **Olha Yaroshko**, Taras Pasternak, Eduardo Larriba, José Manuel Pérez-Pérez (2023). Optimization of callus induction and shoot regeneration from tomato cotyledon explants. *Plants* **12,** 2942. doi: 10.3390/plants12162942**.**
- Eduardo Larriba, **Olha Yaroshko**, José Manuel Pérez-Pérez (2024). Recent advances in tomato gene editing. *International Journal of Molecular Sciences* **25**, 2606. doi: 10.3390/ijms25052606**.**

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Article

Optimization of Callus Induction and Shoot Regeneration from Tomato Cotyledon Explants

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Abstract: Cultivated tomato (*Solanum lycopersicum* L.) is one of the most important horticultural crops in the world. The optimization of culture media for callus formation and tissue regeneration of different tomato genotypes presents numerous biotechnological applications. In this work, we have analyzed the effect of different concentrations of zeatin and indole-3-acetic acid on the regeneration of cotyledon explants in tomato cultivars M82 and Micro-Tom. We evaluated regeneration parameters such as the percentage of callus formation and the area of callus formed, as well as the initiation percentage and the number of adventitious shoots. The best hormone combination produced shoot-like structures after 2–3 weeks. We observed the formation of leaf primordia from these structures after about 3–4 weeks. Upon transferring the regenerating micro-stems to a defined growth medium, it was possible to obtain whole plantlets between 4 and 6 weeks. This hormone combination was applied to other genotypes of *S. lycopersicum*, including commercial varieties and ancestral tomato varieties. Our method is suitable for obtaining many plantlets of different tomato genotypes from cotyledon explants in a very short time, with direct applications for plant transformation, use of gene editing techniques, and vegetative propagation of elite cultivars.

Keywords: in vitro culture; de novo shoot formation; cytokinin; auxin; *Solanum lycopersicum* L.

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1. Introduction

Tomato (*Solanum lycopersicum* L.) is the most widely grown vegetable in the world. According to the latest FAOSTAT report [1], world tomato production exceeded 189 million tons in 2021, with China, India, and Turkey being the largest producers. Global demand for tomatoes is expected to increase in the future. However, in many of the current tomato growing areas, the consequences of human-induced climate change are expected to negatively affect crop productivity due to an increase in average temperature and irregular rainfall in these areas [2]. Therefore, the development of new tomato cultivars with greater tolerance to abiotic (i.e., drought, temperature) and biotic stresses is a priority breeding goal that can be accelerated using new genome editing strategies (i.e., CRISPR/Cas-based approaches) [3]. Successful implementation of these strategies requires the regeneration of whole plantlets from tissue explants, a process that generally relies on hormonal induction of de novo shoot formation from tissue explants. The efficiency of regeneration depends on several factors, including the type of explant, the culture conditions and composition of the regeneration medium, and the genotype and physiological state of the mother plant used [4].

In recent years, the dwarf tomato cultivar Micro-Tom (MT) has been established as a model for functional genomics research due to its small size and short life cycle [5], which allows it to be grown indoors at high densities, and the implementation of high-throughput genetic approaches such as publicly available mutant collections, efficient *Agrobacterium tumefaciens*-mediated transformation protocols, mapping-by-sequencing, and precise genome editing [6,7]. Other classic tomato cultivars that have been widely used in research are M82 and Moneymaker (MM). These three cultivars concentrate most of the

genetic resources available in tomatoes, facilitating their use as experimental models [3]. However, despite the remarkable morphological differences among these three cultivars due to background-specific developmental mutations, genetic diversity in modern cultivated tomatoes is limited. In a recent study, Mata-Nicolás et al. (2020) characterized a collection of diverse *S. pimpinellifolium*, *S. lycopersicum* var. *cerasiforme*, and *S. lycopersicum* var. *lycopersicum* that represent the genetic and morphological variability of tomato at its centers of origin and domestication [8].

Shoot regeneration of various tomato cultivars and wild tomato species by indirect organogenesis (i.e., callus) has been widely reported, with contrasting results due to strong genotype dependence [9]. Indeed, it was recently found that enhanced in vitro shoot regeneration of the wild relative *S. pennellii* over commercial cultivars such as MT depends on three genomic regions of *S. pennellii*, one of which is associated with enhanced expression of the shoot-related genes *WUSCHEL* (*WUS*) and *SHOOT MERISTEMLESS* (*STM*) [10]. In recent years, several reports have been published on the implementation of cost-effective and reproducible *A. tumefaciens*-mediated transformation systems for different commercial tomato cultivars (M82, MT, Rio Grande, Pusa Ruby, Arka Vikas, etc.), all based on efficient regeneration protocols with different proportions of auxin and cytokinin (CK) in the medium depending on the cultivar used [11–15]. Only a few studies have focused on the systematic optimization of several tissue culture parameters to establish a more efficient and reproducible shoot-regeneration system in tomatoes [16,17].

In this study, we evaluated the effectiveness of several tissue culture parameters for de novo shoot regeneration in different tomato cultivars, including modern tomato cultivars and heirloom tomato genotypes from the centers of origin (Perú) and domestication (México) of tomatoes. We found remarkable differences in both callus formation and de novo shoot induction, which depended on both the position of the explants and the genotype of the mother plant. In addition, we performed a detailed histology characterization of de novo shoot formation in M82 cotyledon explants as an example of a tomato genotype with a high in vitro regeneration ability.

2. Results

2.1. Optimization of Callus and Shoot Formation in M82 and MT Cotyledon Explants

We found that the area of tomato cotyledon explants increased approximately threefold during the experiment in indole-3-acetic acid (IAA)-dependent manner (p-value = 0.000; multifactorial ANOVA [MANOVA]; n = 259; Table S1A), but with a non-significant effect of zeatin (ZT) levels (p-value = 0.453) and/or genotype (p-value = 0.207). Callus formation was dependent on both IAA and ZT levels (p-value = 0.000; MANOVA; n = 584), with a small effect of genotype (p-value = 0.071). In MT, callus formation in cotyledon explants incubated without IAA was observed in a lower proportion of explants than in M82, except at high ZT (3 mg/L) (Figure 1a). On the other hand, callus growth, measured as callus area at the end of the experiment, was slightly dependent on exogenous ZT levels (p-value = 0.031; MANOVA; n = 186) and on the interaction between ZT and IAA (p-value = 0.042) (Figure 1b). Indeed, high ZT levels (3 mg/L) produced significantly smaller calluses in both genotypes (p-value = 0.001). We found a positive and significant correlation between callus area and explant growth (r = 0.593; p-value = 0.000) (Figure 1c), which is consistent with the increase in callus area accounting for most of the explant growth. Calluses were mainly formed at the cut ends of the explants. In this region, the callus tissue formed had a creamy yellow color and a compact structure protruding from both abaxial and adaxial sides of the explant (Figure 1d). Next, additional tissue outgrowth was observed near the cotyledon margin and the midrib, which later extended into the whole lamina (Figure 1e). Based on the external appearance of the tissue, we distinguished between soft callus and dense callus, the former being friable in nature and separable by light touch with tweezers.

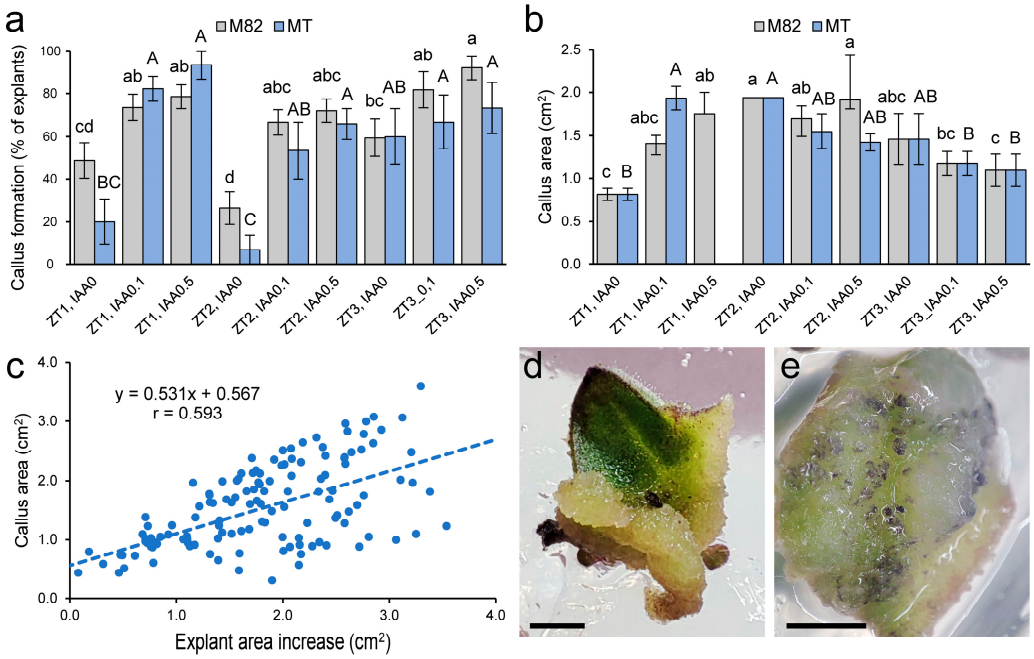

Figure 1. Hormone-induced callus formation in M82 and MT tomato cultivars. (**a**,**b**) Callus formation (**a**) and callus area (**b**) in response to ZT and IAA treatment. Bars indicate mean and standard error of the mean (SEM). Letters indicate significant differences between treatments for a given genotype (p-value < 0.01; Fisher's least significant difference [LSD]). Raw data is shown in Table S1. (**c**) Correlation between explant and callus area. (**d**,**e**) Representative images of callus formation in distal (**d**) and proximal (**e**) cotyledon explants at 21 days. Scale bars: 3 mm.

Interestingly, we found striking differences in de novo shoot formation from these calluses depending on genotype and exogenous ZT levels (p-value = 0.000; MANOVA; n = 383). Shoot primordia formation was negatively correlated with increasing ZT levels in both genotypes, and M82 showed a significantly higher proportion of explants with prominent shoot primordia than MT (Figure 2a), where only 20% of explants were able to develop shoot primordia (p-value = 0.000; n = 383). In addition, the number of shoot primordia formed per explant at the end of the experiment was also strongly dependent on genotype (p-value = 0.003; n = 217; MANOVA), with M82 (9.3 ± 6.4 shoot primordia per explant; n = 188 explants) showing about a three-fold increase in the number of shoots formed compared to MT (3.4 ± 1.8; n = 29) (Figure 2b). Our results indicate that the best hormonal combination for callus growth in M82 and MT cotyledon explants was 1 mg/L of ZT and 0.1 mg/L of IAA (1.72 ± 0.73 cm^2; n = 57; p-value = 0.002; LSD), whereas the best hormonal combination for higher induction of shoot formation was 1 mg/L of ZT and 0.5 mg/L of IAA (12.8 ± 9.7 shoot primordia per explant; p-value = 0.000; LSD; n = 73) (Figure 2c,d). In M82, primordia were observed early as darker filiform protrusions from the adaxial surface, with a higher frequency in a region close to the proximal incision where the dense callus develops (Figure 2e). Some spatial and temporal arrangement of these structures was observed at early time points (Figure 2f), although not all these primordia develop into functional shoots (Figure 2g,h), suggesting internal competition among primordia as a limiting factor necessary for subsequent shoot growth. Then, primordia develop near the distal incision and from the abaxial surface of the explant, as well as from inner regions of the lamina in those explants with homogeneous dense callus formation (Figure S1A,B). In MT cotyledon explants, both primordia formation and functional shoot establishment was severely reduced in comparison to M82 (Figure 2a–d). In MT, these shoot primordia were more widely spaced, developed at a slower rate, and exhibited a lighter green color than in M82 (Figure S1C). From our studies, the best hormonal combination for callus growth was

1 mg/L ZT and 0.1 mg/L IAA, and for higher induction of shoot formation, it was 1 mg/L ZT and 0.5 mg/L IAA.

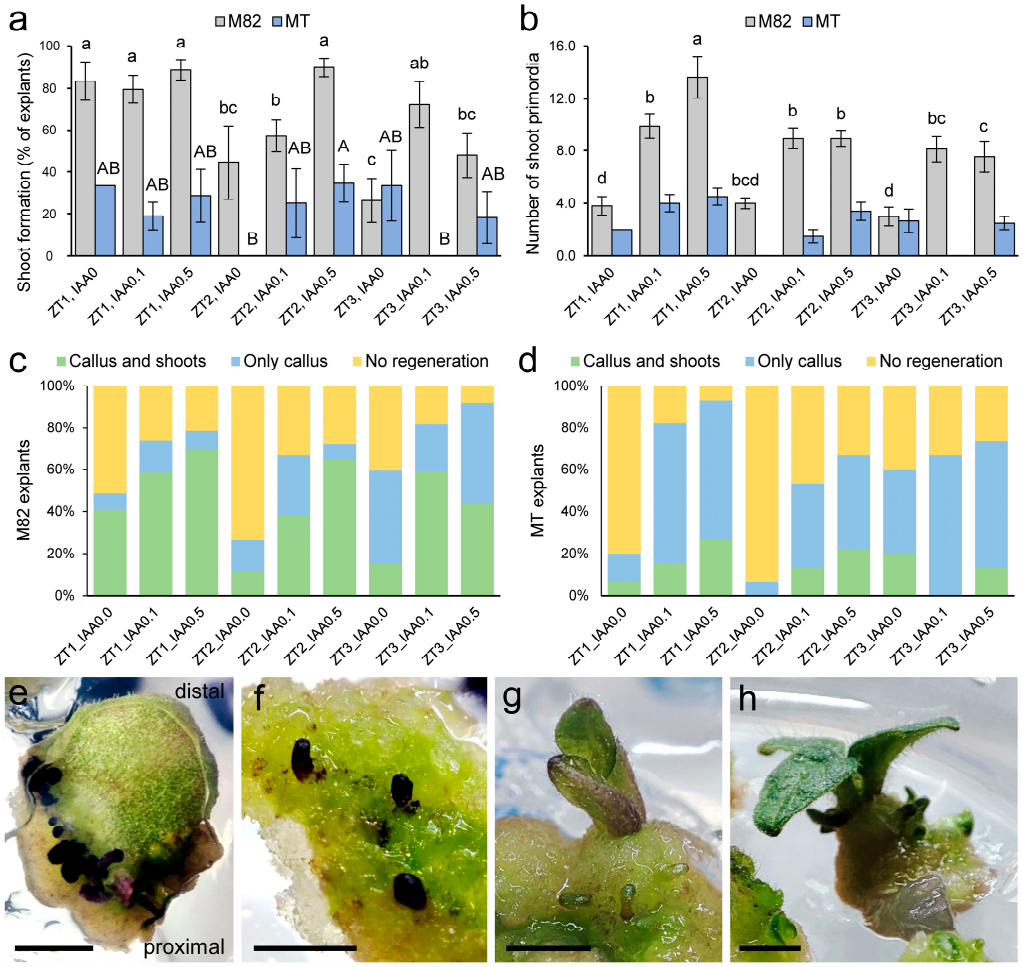

Figure 2. Hormone-induced shoot formation in M82 and MT tomato cultivars. (**a**,**b**) Shoot formation (**a**) and number of shoot primordia (**b**) in response to ZT and IAA treatment. Bars indicate mean and SEM. Letters indicate significant differences between treatments for a given genotype (p-value < 0.05; LSD). Raw data is shown in Table S1. (**c**,**d**) Regenerative response of M82 (**c**) and MT (**d**) explants in response to ZT and IAA treatment. (**e**–**h**) Representative images of a time series for shoot formation in M82 cotyledon explants at 21 (**e**,**f**), 25 (**g**), and 55 (**h**) days. Scale bars: 3 mm.

2.2. Evaluation of the Regenerative Potential of Explants in Different Tomato Genotypes

To investigate whether the regenerative potential of the explants depends on the region of the cotyledon used, we cut 7-day-old cotyledons into three regions of similar size: apical, central, and basal (Figure S2A). We found significant differences in explant area according to genotype (p-value = 0.000; MANOVA; n = 420), consistent with the larger size of cotyledons in the BGV016054, M82, and MM genotypes (Table S1b and Figure S2B). The increase in explant area during the experiment was significantly dependent on genotype and region of the cotyledon used (p-value = 0.000; MANOVA; n = 409; Table S1B and Figure S2B), with M82 showing the greatest increase in explant area, and the apical region of the explants showing the least growth in most genotypes. Callus formation was dependent on genotype and region of the cotyledon used (p-value = 0.000; MANOVA; n = 409; Figure 3a), with a weaker and positive effect of ZT levels (p-value = 0.018; Table S1B). Overall, callus formation reached ~40% in apical explants and ~70% in basal explants. Interestingly, 2 mg/L ZT positively increased callus formation only in apical explants and had no effect in central and basal explants. We also found that callus formation was significantly reduced (p-value

= 0.000; LSD; n = 409) in explants smaller than 0.13 cm^2 compared to intermediate size explants (Table S1B), suggesting that a minimum explant size is required for functional regeneration in tomatoes. BGV007927 and BGV016054 showed the lowest levels of callus formation, whereas M82 and MM showed the highest levels of callus formation from cotyledon explants, mainly from the central and basal regions of the cotyledon (Figure 3a).

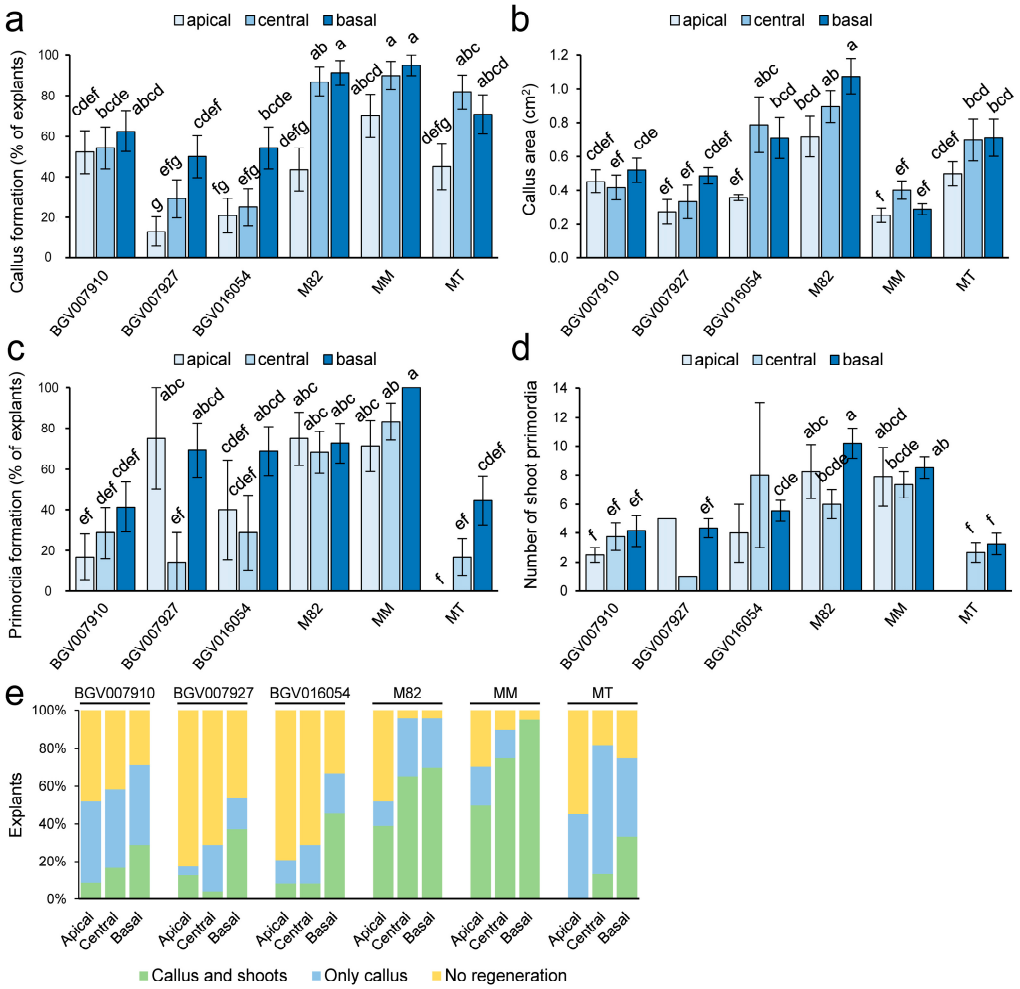

Figure 3. Hormone-induced callus and shoot formation is genotype and region dependent. (**a**,**b**) Callus formation (**a**) and callus area (**b**) in the studied genotypes from different regions of the cotyledon. (**c**,**d**) Shoot formation (**c**) and number of shoot primordia (**d**) in the studied genotypes from different regions of the cotyledon. Bars indicate mean and SEM. Letters indicate significant differences between samples (p-value < 0.01 (**a**,**c**) or p-value < 0.05 (**b**,**d**); LSD). Raw data are shown in Table S1. (**e**) Regenerative response of the studied genotypes with respect to the different regions of the cotyledon.

We determined callus emergence visually and found that M82 showed significantly lower values (15.0 ± 2.0 days; n = 49) than the other genotypes (p-value = 0.000; LSD; n = 225), with the higher delay in callus emergence shown by BGV007927 (18.9 ± 1.4 days; n = 27). Interestingly, in M82, callus emergence occurred slightly earlier in apical than in basal explants (Table S2). We used callus area as an estimate of callus growth during the experiment, and this trait was significantly dependent on genotype (p-value = 0.000; MANOVA; n = 229; Figure 3b). In addition, callus growth was also influenced by the region of the cotyledon used as an explant, with callus areas increasing from apical to basal explants in some genotypes (p-value = 0.006), such as M82 and BGV016054 (Figure 3b). Similarly, we also found a slightly positive and significant correlation between callus area

and explant growth (r = 0.451; *p*-value = 0.000; Table S1B). Despite the high level of callus formation in MM, callus size was smaller than in MT (Figure 3b).

The time of de novo primordia formation was determined visually and found to be significantly influenced by genotype (*p*-value = 0.000; MANOVA; *n* = 135) but not by region (*p*-value = 0.207) or area of the explants (*p*-value = 0.060; Table S2 and Figure S3). Consistent with early callus formation in M82, this genotype showed the fastest initiation of primordia (17.3 ± 0.6 days; *n* = 40), whereas BGV007927 showed the greatest delay (27.5 ± 1.0 days; *n* = 13). These results suggest that in this later genotype, there is a delay of approximately 8.5 days between callus initiation and de novo primordia formation as compared to M82 (Table S1b). On the other hand, the proportion of explants forming primordia was dependent on genotype (*p*-value = 0.000; MANOVA; *n* = 247) and the region of the cotyledon used as an explant (*p*-value = 0.001; Figure 3c). MM showed the highest level of de novo primordia formation (~86%), whereas MT showed the lowest level of primordia formation (~24%). Consistently, basal explants showed the highest proportion of primordia formation among the genotypes studied (*p*-value = 0.007; LSD). Interestingly, the number of primordia formed was genotype dependent (*p*-value = 0.000; MANOVA; *n* = 134), with a slight effect of explant region (*p*-value = 0.087) but little contribution from ZT levels (*p*-value = 0.624). MT produced the lowest number of primordia per explant, 3.6 ± 2.4 (*n* = 11), while M82 and MM produced the highest number of primordia: 9.9 ± 5.6 (*n* = 38) and 10.1 ± 6.3 (*n* = 44) primordia per explant, respectively (Figure 3d). We found no significant correlation between explant size and number of primordia (r = −0.069; *p*-value = 0.427; Table S1b). We found that most of these primordia developed into shoot-like structures in less than one week (r = 0.978; *p*-value = 0.000). Consistent with these results, the number of shoot primordia was also genotype dependent (*p*-value = 0.000; MANOVA; *n* = 134), with a small effect of the explant region (*p*-value = 0.061) but no direct contribution of ZT levels (*p*-value = 0.838) (Table S1B), like that described above. In summary, callus formation was dependent on the explant region, ranging from ~40% in apical explants to ~70% in basal explants. M82 and MM were the two genotypes with high levels of callus and primordia formation. In this sense, M82 was the best genotype in primordia initiation, while MM presented the highest de novo primordia formation. For these parameters, cultivar MT showed the lowest values.

The presence of dense calluses was detected in most of the genotypes studied (BGV016054, BGV007910, M82, MM, and MT), while BGV007927 formed soft calluses from the beginning. The location of the callus induction showed some genotype-dependent differences (Figure 4). The location and structure of M82 and MT callus were mentioned above. MM formed calluses on both basal and apical sections of explants (Figure 4a,c). These calluses were small and light green in color. In addition, the explants of MM developed many round or oval callus regions of small size and whitish color within the lamina, in both the adaxial and abaxial surfaces (Figure 4b,c). BGV016054 and BGV007910, like M82, formed callus tissue mainly in the basal region near the cut end (Figure 4(d′,e′)). Small globular areas of whitish callus were detected on the adaxial side of both genotypes (Figure 4d,e). BGV007927 initially formed pale green or whitish callus tissue in the cut region, both apical and basal, and tends to transform almost the entire explant into callus tissue after some time in the culture medium. The first morphological structures observed in the callus tissue were round and protruded from the surface of the lamina and were probably the foci of formation of the new organs, which we will refer to as the globular phase (arrowheads in Figure 4). These globular structures continued to elongate and acquired an oval structure during the so-called filiform phase. Later, the filiform structures were transformed into small leaf primordium nodes, as indicated by the presence of newly developed glandular trichomes on their epidermis (asterisks in Figure 4). The color of the globular structures ranged from dark purple or almost black (M82; Figure 4f(f′)) to green (MT) or light green (BGV007910 and BGV016054; Figure 4d,e). These structures displayed variable color in MM and BGV007927 explants. The detailed localization of the globular structures and filiform structures of M82 (Figure 4g) and MT has been described above. MM, BGV007910,

and BGV016054 structures were located near the cut ends and formed clusters. BGV007927 also initially formed primordia (single/pairs or small clusters) from the cut basal end of explants, but after about 1 month, when most explants became callus tissue, the primordia were randomly localized and associated with the callus site. Some of the shoot primordia became a functional meristem and produced stems available for rooting.

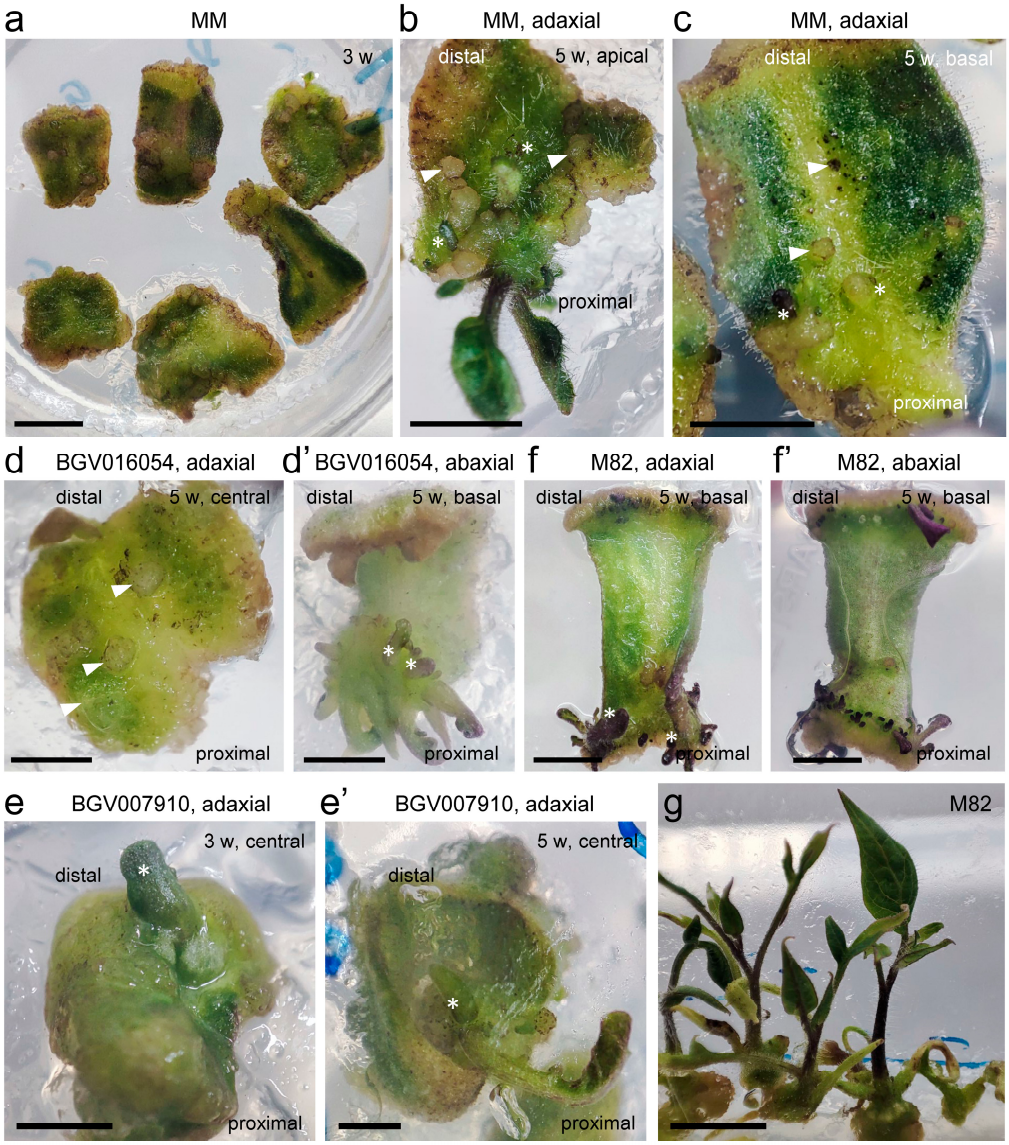

Figure 4. De novo organ formation in excised cotyledon explants of some of the genotypes studied. (**a**–**c**) MM [representative examples of apical (**b**) and basal (**c**) explants are shown] at 32, 39, and 24 days, respectively; (**d**,**d′**) BGV016054 at 19 days, (**e**,**e′**) BGV007910 at 24 and 19 days, respectively, and (**f**,**f′**) M82 at 21 days. (**d**,**f**): adaxial side; (**d′**,**f′**): abaxial side; arrowheads point to the globular structures within the callus tissue, and asterisks indicate some of the developing leaf primordia. (**g**) M82 explants with elongated shoots before rooting at 80 days. Scale bars: 3 mm.

2.3. Cellular Features of De Novo Organ Formation in Tomato Cotyledon Explants

Due to the heterogenous response in callus and shoot morphology among the tomato genotypes studied, we decided to investigate the cellular characteristics that occur during de novo organogenesis using M82 basal cotyledon explants. As mentioned above, we observed that many leaf primordia were initiated at a certain distance from the basal incision and with a very regular spatial and temporal pattern (Figure 5a).

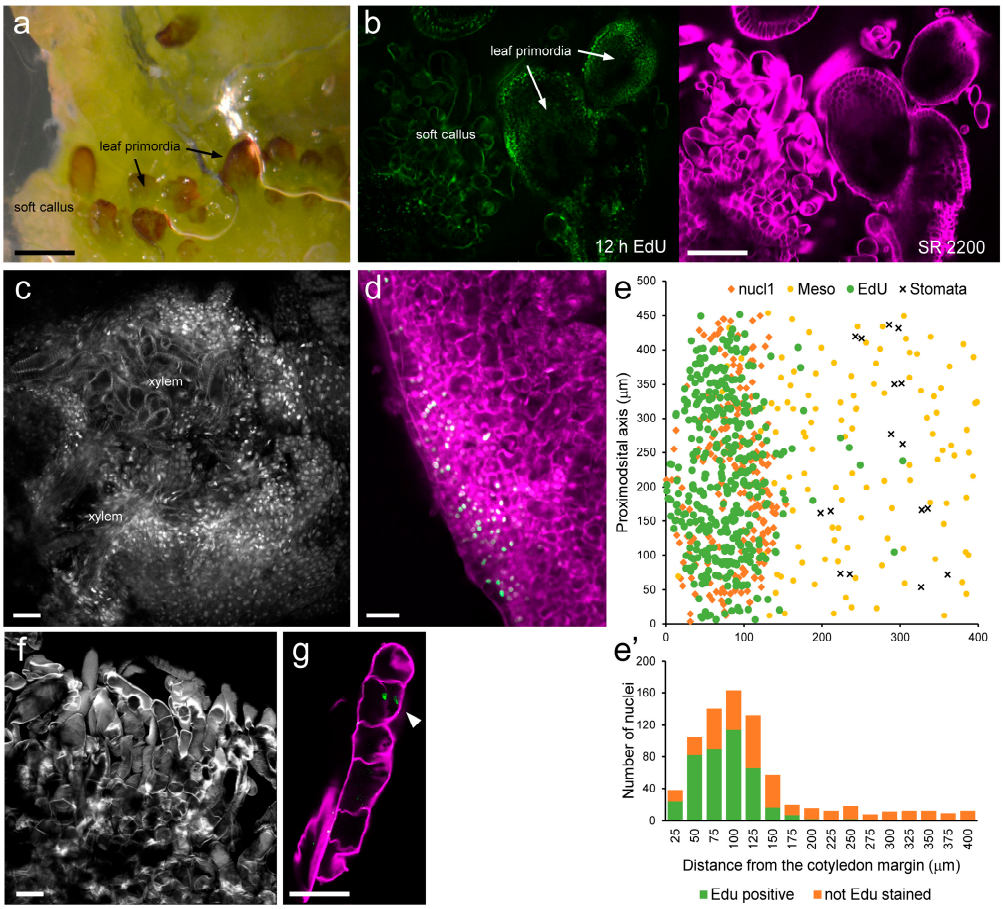

Figure 5. Cellular features of de novo organ formation in tomato cotyledon explants. (**a**) Detailed observation of hormone-induced shoots in M82 cotyledon explants at 3 weeks, showing soft callus tissue and incipient leaf primordia. (**b**) Microscopic observation of the explant shown in (**a**), incubated for 12 h with 5-ethynyl-2′-deoxyuridine (EdU; green) and stained for cell walls with SR2200 (magenta). (**c**) Detailed observation of a globular callus tissue from (**a**) stained with DAPI, where xylem cells are clearly observed. (**d**) Details of the cotyledon margin in M82 cotyledon explants at 4 days on regeneration medium, incubated for 4 h with EdU (green) and stained with DAPI (magenta). (**e**) Nuclear coordinates from (**d**,**e′**) histogram of the percentage of EdU-positive cells. Nuclei and EDU positive nuclei were detected and plotted in Cartesian coordinates. (**f**,**g**) Microscopic observation of soft callus tissue (**g**) and details of a dividing cell (EdU, green, white arrow; SR2200, magenta). Scale bars: 2 mm (**a**) and 40 µm (**b**–**g**).

In order to localize cell cycle/DNA replications activity, we have used uridine analogue EdU, which is incorporated into nuclei exclusively during DNA replication and can be easily detected and quantified. SCRI Renaissance Stain 2200 (SR2200) was labeled cell wall (cellulose) and has been used to visualize and quantify cell structure, while DAPI has been used for visualization of cell nuclei and chromatin organization. Compared to neighboring soft callus tissues, cells in these incipient leaf primordia were characterized by smaller size and active DNA replication, as indicated by the strong staining of their nuclei with EdU (Figure 5b and Figure S4A–C). Another interesting observation was that the isolated calli found on the cotyledon lamina consisted of many dividing cells on the surface and xylem cells in the inner tissues (Figure 5c and Figure S4D,E). The callus tissue at the cotyledon margin consisted of small isodiametric cells with high EdU staining of their nuclei (Figure 5d), indicating their meristematic potential. A cell division gradient from the cotyledon margin to the inner lamina was clearly observed (Figure 5e). On the other hand, the soft callus tissue was characterized by large cells with brightly stained cell walls (Figure 5f), which occasionally divided (Figure 5g). Taken together, our results

indicate differential tissue-specific responses in tomato explants to the addition of external hormones that merit further investigation.

3. Discussion

In this work, we optimized the protocol for hormone-induced shoot regeneration in tomato cotyledon explants using two widely used tomato genotypes, M82 and MT, and four other cultivars with highly divergent genetic backgrounds (see Section 4). We found that tomato cotyledon explants, regardless of their genotype and their regenerative responses, increased in size during the experiment, indicating that they contain sufficient resources (hormones, photosynthates, etc.) to maintain autonomous growth of the explants for several weeks, in contrast to what occurs in *Arabidopsis thaliana* cotyledons, whose postembryonic growth depends mainly on cell expansion [18].

Previous studies in *A. thaliana* have shown that treatment with the exogenous CKs 6-benzylaminopurine (BAP) or ZT induces endogenous IAA biosynthesis and increases steady-state auxin levels in young shoots and roots [19]. Addition of CKs can also affect endogenous auxin distribution by regulating the PIN-FORMED (PIN) auxin efflux transporters [20]. Our results on callus and adventitious shoot formation in tomato cotyledon explants in response to exogenous treatment with ZT and IAA also suggest a complex regulation of endogenous auxin and CK levels. Interestingly, exogenous auxin significantly enhanced the effect of ZT on callus formation and de novo shoot formation, whereas higher levels of ZT negatively affected de novo shoot formation. Indeed, in the absence of IAA, high levels of ZT (3 mg/L) significantly enhanced the callus formation response in the MT background but negatively regulated callus growth in this genotype, as this is highly dependent on the endogenous auxin-to-CK ratio, as described in other *Solanaceae* species [21]. At low ZT levels (1 mg/L), adventitious shoot production in M82 was dependent on increasing exogenous IAA levels. However, the higher regenerative response of M82 over MT in terms of de novo shoot induction could be because the MT background contains mutations related to gibberellin (GA) signaling and brassinosteroid biosynthesis [5], which could directly affect shoot apical meristem (SAM) activity, as has recently been shown for the positive role of GA in SAM growth in *A. thaliana* [22].

In a previous report, the addition of 0.05 or 0.1 mg/L IAA to ZT-containing growth medium reduced the recovery time of regenerated M82 plantlets by 6 weeks [13]. In another work, plant regeneration by indirect organogenesis was developed for 7-day-old cotyledon explants of four tomato cultivars (Rio Grande, Roma, hybrid 17905 and M82) in Murashige and Skoog (MS) medium supplemented with different combinations and concentrations of plant growth regulators (PGRs) [16]. Regenerating calli resulted in the formation of multiple shoots after 3 weeks on medium containing 3 mg/L BAP and 0.1 mg/L IAA. Furthermore, Sandhya et al. (2012) reported that 2.0 mg/L ZT in combination with 0.1 mg/L IAA produced the highest number of shoots from the cotyledon explants of two different tomato cultivars, Arka Vikas and Pusa Early Dwarf [23]. Shorter explant-to-plantlet regeneration time is always desirable, as it reduces labor and resource costs and allows high-throughput approaches for early recovery and evaluation of transgenic lines. These and other results [24] suggest a genotype × environment (i.e., PGRs) interaction in the regenerative response of tomato cotyledon explants. We speculate that the addition of IAA to the growth medium facilitates auxin and CK crosstalk within the explants, which is then required for the acquisition of competence to establish de novo shoot apical meristems by specific developmental regulators [25]. In our work, we avoided the use of 2,4-dichlorophenoxyacetic acid, which is routinely used in indirect somatic embryogenesis but is known to cause morphological abnormalities in different species because of physiological disorders or somaclonal variation [26].

We investigated the effect of the region of the cotyledon (basal, central, and distal) used as explants on callus formation and de novo shoot induction in six tomato genotypes. In all cases, callus growth was observed at both cut ends (proximal and distal) of each explant, shortly after excision, and with greater growth of callus tissue in the proximal

region of the explants regardless of the region of the cotyledon used. Callus growth in the proximal region of the explants is likely dependent on endogenous auxin gradients that are dynamically established by the endogenous PIN-mediated polar auxin transport system, as previously demonstrated in tomato hypocotyl explants [27,28]. Callus formation is achieved in all six genotypes at different rates with a region × genotype dependence: in general, the basal region of the cotyledon is more responsive than the apical one, and the studied heirloom tomato cultivars are less responsive than the commercial ones. The minimum size of explants that produced effective regeneration under our conditions was approximately 0.20 cm^2, and the average time for callus establishment ranged from 15 to 19 days. De novo shoot primordia formation was strongly genotype dependent, with MM showing the highest number of fully developed shoot primordia. For most genotypes, explants from the basal region of the cotyledon showed the highest number of developed shoot primordia. In addition to MT, two heirloom varieties from the Varitome collection [7] that are from México, BGV007910 and BGV007927, showed a low proportion of adventitious shoots formed. These results suggest interesting genotype-dependent regenerative responses in the Varitome collection that deserve further investigation.

Previously, Chaudry et al. (2010) [29] investigated the regenerative capacity of hypocotyl and leaf discs from MM explants, using a combination of ZT and IAA, which induced de novo shoot formation from hypocotyl explants but not from leaf discs. Lee et al. (2020) found that the optimal culture conditions for de novo shoot formation from MT explants were MS mediums containing 1 mg/L ZT, 0.1 mg/L IAA, and 3% sucrose. They also found non-significant differences in de novo shoot formation with respect to explant age up to 10 days after germination. Consistent with our results for several tomato genotypes (see above), de novo shoot formation in MT was much higher in the basal cotyledon explants than in the central and apical explants, with a greater positive effect on whether the abaxial side of the explant touched the medium [16]. Interestingly, the region of the hypocotyl closest to the cotyledons produced the highest number of de novo shoots, which correlated with higher expression of the shoot identity genes WUS and STM in this region [16]. In a recent investigation, Sundhya et al. (2022) [30], using cotyledon explants from two Indian tomato cultivars, demonstrated that ZT was the most effective CK for shoot formation compared to BAP or thidiazuron. Taken together, our results indicate that the optimal conditions for de novo shoot formation in tomatoes are the use of the basal segment of young cotyledons (7-day-old) placed in culture medium (MS, 1 mg/L ZT, 0.1 mg/L IAA, 2% sucrose) on their abaxial surface.

The callus tissues formed in tomato explants in response to treatment with ZT and IAA were heterogeneous in nature. Macroscopically, we distinguished between soft or friable calli, usually found at the edges of the explants and near the cut region, and dense calli, the latter composed of small, isodiametric, and actively dividing cells. Soft calli are composed of multicellular structures resembling trichomes. These structures grow polarly outward from the explant surface by limited cell division and mainly by cell expansion. This soft callus tissue is disorganized and rarely produces adventitious shoots. In some of the genotypes studied, such as BGV0016054 or MT, the soft callus tissue prevailed.

Dense calli are normally found in the cotyledon margin and within the cotyledon lamina. Interestingly, a decreasing cell division gradient in palisade mesophyll cells is observed from the cotyledon margin, as evidenced by the EdU/DAPI nuclear ratio (this work). These results suggest that cells at the cotyledon margin retain their meristematic potential. Some mesophyll cells adjacent to this tissue also replicate DNA but rarely divide, resulting in cell growth by endoreduplication. In *A. thaliana*, a complex regulation of transcription factors contributes to leaf margin growth, although the molecular mechanism remains to be elucidated [30,31]. We speculate that exogenous ZT could activate the marginal meristem of tomato cotyledons, probably through the WUSCHEL-RELATED HOMEOBOX1 (WOX1) and WOX3 transcription factors, which in *A. thaliana* are known to positively regulate the expression of *YUCCA1* (*YUC1*) and *YUC4* to synthesize auxin in the margin and thus control lamina outgrowth [32]. Dense calli found on the cotyledon

lamina are highly structured, with many dividing cells in the epidermis and palisade mesophyll resembling immature shoot meristems, while new xylem cells form in the spongy mesophyll just below, presumably by hormone-mediated transdifferentiation of these cells [33]. These results suggest that within dense calli, some compartmentalization of CK and auxin responses is expected, leading to cell cycle activation in the epidermal/palisade mesophyll cells and near the margin, and increasing gradients of cell differentiation from the margin to the cotyledon lamina as well as from the epidermal/palisade mesophyll cells into the spongy mesophyll cells in response to newly formed adaxial-abaxial and mediolateral auxin gradients. The availability of *DR5:mScarleti-NLS* and *TCSn:mNeonGreen-NLS* tomato plants [34] will allow precise observation of endogenous auxin and CK response gradients during callus formation and de novo shoot induction, which will help clarify our histological observations.

A competitive balance between callus formation and shoot regeneration has been proposed. In *A. thaliana* explants, callus tissue resembles a root primordium with active auxin signaling [35]. However, shoot regeneration is dependent on CK signaling as well as the repressing of root identity [36]. Multiple shoots were initiated at both the adaxial and abaxial surfaces of the dense callus tissue, mainly near the basal region of the cotyledon explants but not at the cutting edge where soft callus tissue is formed. In analogy to the regeneration model for tomato hypocotyl explants [27,28,37], wound-induced local auxin biosynthesis and endogenous polar auxin transport pathways might create an auxin gradient that is maximal at the cut end of the explants, leading to soft callus production. In the presence of ZT, a high CK-to-auxin ratio is established in the region distal to the cut end of the explants, which may be sufficient to induce dense callus formation with SAM identity foci. A similar scenario could explain the formation of shoot primordia within the globular callus on the cotyledon lamina, where cells near the surface actively divide in response to the CK treatment and establish new auxin sinks in the inner mesophyll, causing transdifferentiation of spongy mesophyll cells into xylem bundles that establish new polar auxin transport routes from the dividing cells of the callus to the internal vasculature, generating a minimum of auxin in some of the dividing cells at the adaxial side that could contribute to the specification of new SAMs in this region.

Comparison of our study with [38] demonstrated that our hormone combination (ZT/IAA) can provide a greater number of shoots in the MM cultivar. Moreover, our conditions also showed a greater number of the shoots per explants as previously reported [39].

In conclusion, our study extends the knowledge of the regenerative capacity of cotyledon explants through the analysis of three tomato cultivars widely used in research and three ancestral tomato lines from the Varitome collection. In this sense, we have determined the optimal concentrations of 1 mg/L ZT + 0.5 mg/L IAA, as well as the regions of the cotyledon that showed the highest rate of regeneration. Finally, we performed a histological study of de novo shoot formation, which adds to the knowledge of this process. Our presented method has the advantage of being carried out in 6-well plates, which allows high throughput escalation, as well as rapid regeneration, and can be applied to different tomato varieties. These advantages are very useful for plant transformation and gene editing experiments.

4. Materials and Methods

4.1. Plant Materials and Growth Conditions

Solanum lycopersicum L. seeds were germinated and cultivated as previously reported [40]. In our experiments, we used three well-known tomato cultivars, M82, MM, and MT, as well as three other lines from the Varitome collection: BGV007910, BGV007927, and BGV016054 [8] (Table 1). For E1, E2, and E3 experiments, 7-day-old cotyledons were cut in half (0.51 ± 0.18 cm^2) and incubated in 6-well plates with MS basal salt medium containing 2% sucrose and 0.8% agar supplemented with ZT (1, 2 or 3 mg/L) or IAA (0, 0.1 and 0.5 mg/L), with the abaxial side in contact with the agar surface. For the E4 experiment, 7-day-old cotyledons were cut into three regions of similar size, apical, central, and basal

(0.27 ± 0.17 cm^2), and incubated in an MS medium containing 2% sucrose and 0.8% agar supplemented with 1 or 2 mg/L ZT and 0.5 mg/L IAA. Cultivation conditions were 16 h LED light/8 h dark period and 23–24 °C for all experiments. Experiments were performed in three biological replications with a minimum of 15 cotyledons per genotype and treatment (E1, E2 and E3; $n = 585$ explants) or with 12 cotyledons per genotype and treatment (E4; $n = 432$ explants). Plates were incubated for 6 weeks, with explants periodically transferred every 2 weeks to freshly prepared media containing a similar combination and concentration of growth stimulants. Plates were scanned weekly, and the presence of calluses and shoot primordia was visually assessed using a stereomicroscope. Explant and callus areas were quantified from scanned images using ImageJ [41] after freehand line drawing. Callus/primordia/shoot emergence was defined as the first day of callus/primordia/shoot observation after daily examination of each explant under a stereomicroscope. Shoots were distinguished from primordia by the presence of glandular trichomes.

Table 1. Tomato genotypes studied in this work.

Accession	Organism		Name	Genotype [1]
LA3475	*S. lycopersicum* var. *lycopersicum*		M82	*sp*; *u*; *obv*; *I*; *Ve*
LA3911	*S. lycopersicum* var. *lycopersicum*		Micro-Tom	*d*; *sp*; *ej-2^{w}*; *u*; *I*; *Sm*
LA2706	*S. lycopersicum* var. *lycopersicum*		Moneymaker	*sp^{+}*; *ej-2^{w}*; *u*; *obv^{+}*
Accession	**Organism**	**Country**	**Site**	**Latitude, Longitude**
BGV007910	*S. lycopersicum* var. *cerasiforme*	México	Palo de Arco; Ciudad Valles; San Luis de Potosí	21.91, 99.16
BGV007927	*S. lycopersicum* var. *cerasiforme*	México	El Vergel: Culiacán. Sinaloa	24.73, 107.79
BGV016054	*S. lycopersicum* var. *cerasiforme*	Perú	Rumizapa	6.45, 76.47

[1] *d*: dwarf; *ej-2^{w}*: enhancer of jointless-2^{weak}; *I*: Immunity to Fusarium wilt; *obv*: obscuravenosa; *obv^{+}*: obscuravenosa$^{clear\ vein}$; *sp*: self-pruning; *sp^{+}*: self-pruning^{+}: self-pruning$^{wild\text{-}type\ allele}$; *Sm*: Stemphyllium resistance; *u*: uniform ripening; *Ve*: Verticillium resistance.

4.2. Statistical Analyses

Statistical analyses of the data and descriptors (mean, SEM, maximum and minimum, and correlation values) were estimated using StatGraphics Centurion XVI version 16.1.03 (StatPoint Technologies, Warrenton, VA, USA). Outliers were identified and were excluded for subsequent analyses, as described elsewhere [42]. To compare data for a given variable, we performed multiple testing analyses using the multivariate ANOVA, F-test, or Fisher's least significant difference post hoc test, as indicated. Significant differences were defined as a significance level of 1% (p-value < 0.01), unless otherwise indicated. To determine the correlation between the different parameters, multiple correlation tests were performed.

4.3. Microscopic Observation and EdU Staining

To visualize the origin of the cell cycle progression, 7- to 10-day-old cotyledon explants grown on regeneration medium for 3–4 days were transferred to liquid medium with the same composition, and EdU was added for the indicated pulse labeling time. Explants were fixed, and EdU was detected as previously described [43]. Samples were stained with DAPI and mounted on slides with double spacers (300 µm thick). To characterize tissue structure, cotyledon explants with de novo shoot primordia were fixed in 4% formaldehyde solution for 30 min, cleaned as described to increase tissue transparency, and partially digested for cell wall and membrane [43,44]. Explants were incubated with the EdU detection solution for 30 min and then incubated in MTSB buffer (pH 8.1) supplemented with 1:1000 SCRI Renaissance 2200 (SR2200; Renaissance Chemicals Ltd., Selby, UK) and mounted on slides with double spacers. Images were captured using a Leica STELLARIS STED microscope (Leica Microsystems, Wetzlar, Germany). For SR2200 and DAPI: ex. 405; for Alexa 488 (EdU): ex: 488. Image analyses were performed as described [44].

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/plants12162942/s1, Figure S1: Hormone-induced shoot formation in M82 and MT tomato cultivars (cont.); Figure S2: Experimental design to determine the effects of genotype and explant region on hormone-induced callus and shoot formation; Figure S3: Timing of callus and shoot formation in individual tomato explants of MT and MM grown under the indicated conditions; Figure S4: Cellular analysis of de novo leaf formation in tomato cotyledon explants; Table S1: Raw data of experiments performed in this work; Table S2: Timing of callus and shoot formation in individual tomato explants of the studied genotypes grown under indicated conditions.

Author Contributions: Conceptualization, E.L. and J.M.P.-P.; methodology, O.Y. and T.P.; formal analysis, E.L. and J.M.P.-P.; investigation, O.Y. and T.P.; resources, J.M.P.-P.; data curation, O.Y.; writing—original draft preparation, O.Y.; writing—review and editing, E.L. and J.M.P.-P.; visualization, J.M.P.-P.; supervision, E.L. and J.M.P.-P.; project administration, J.M.P.-P.; funding acquisition, J.M.P.-P. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: All data generated or analyzed during this study are provided in this published article and its supplementary data files, or they will be provided upon a reasonable request.

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Conflicts of Interest: The authors declare no conflict of interest.

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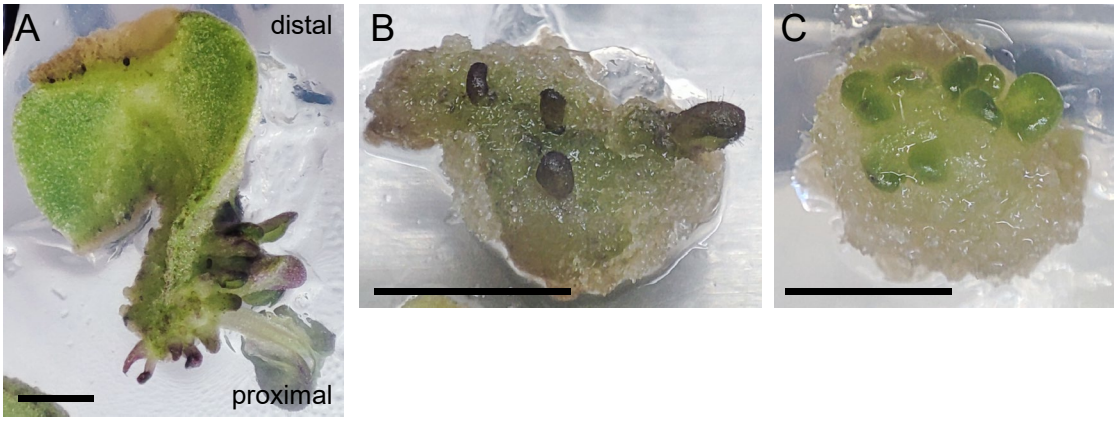
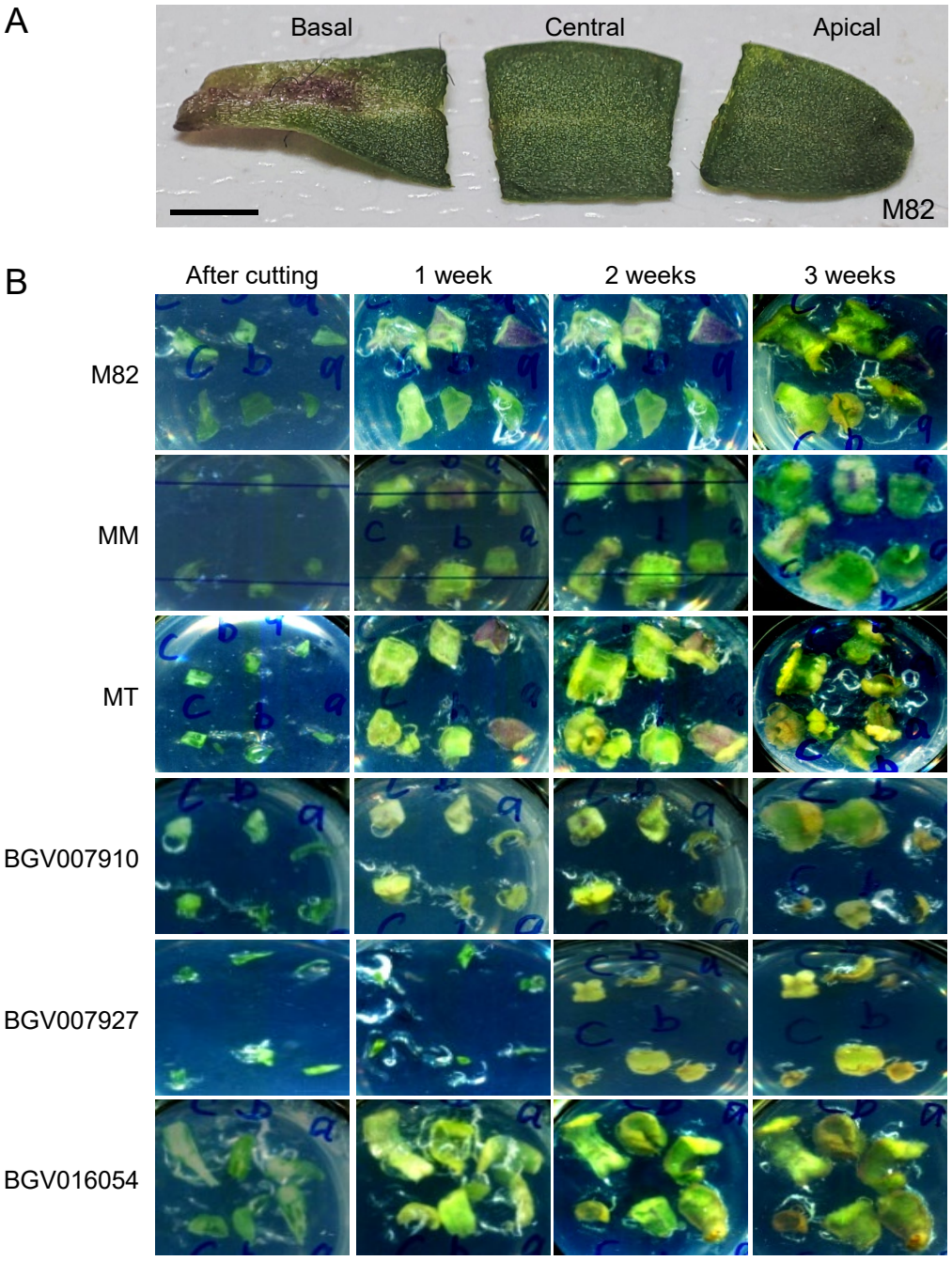
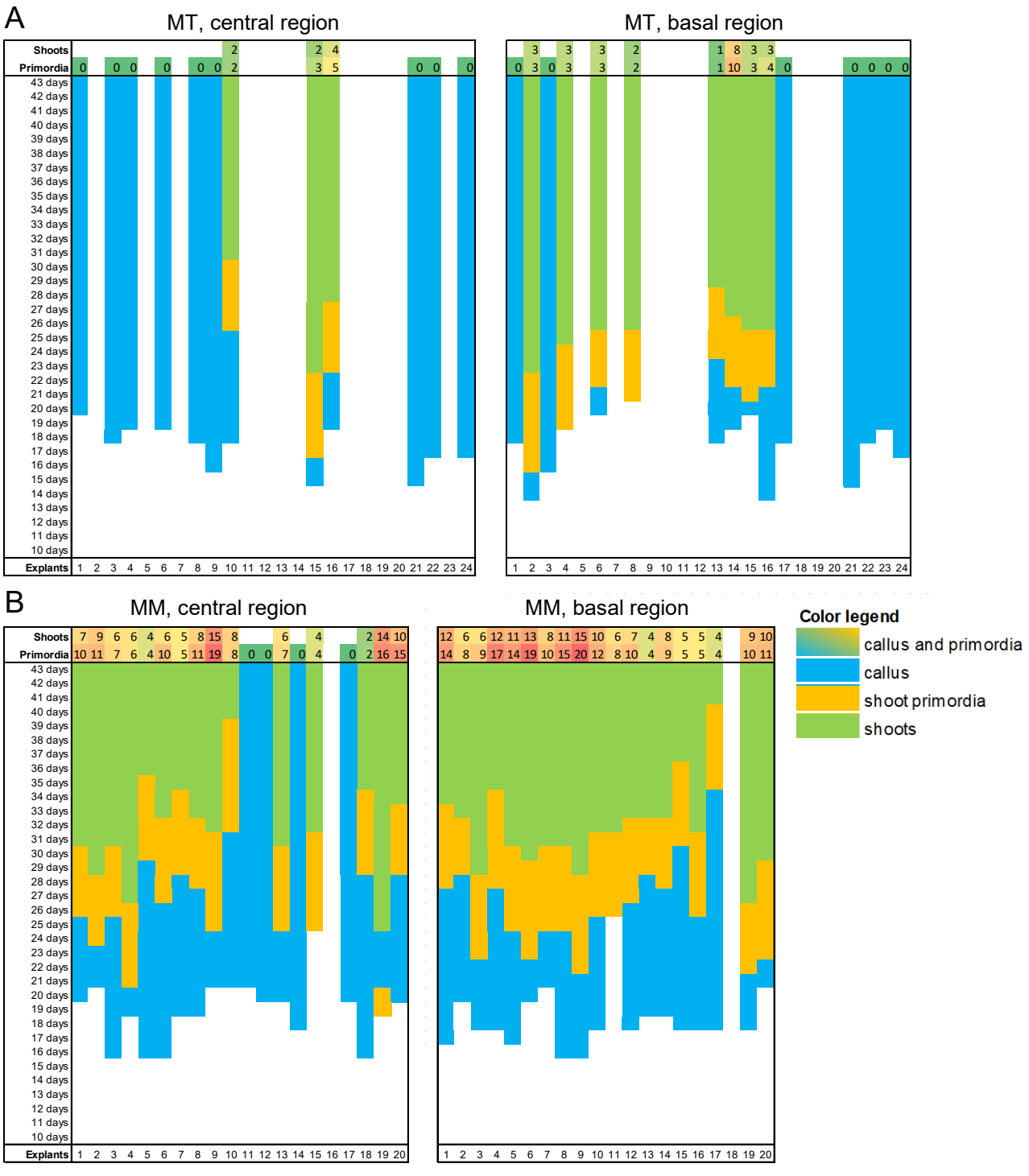


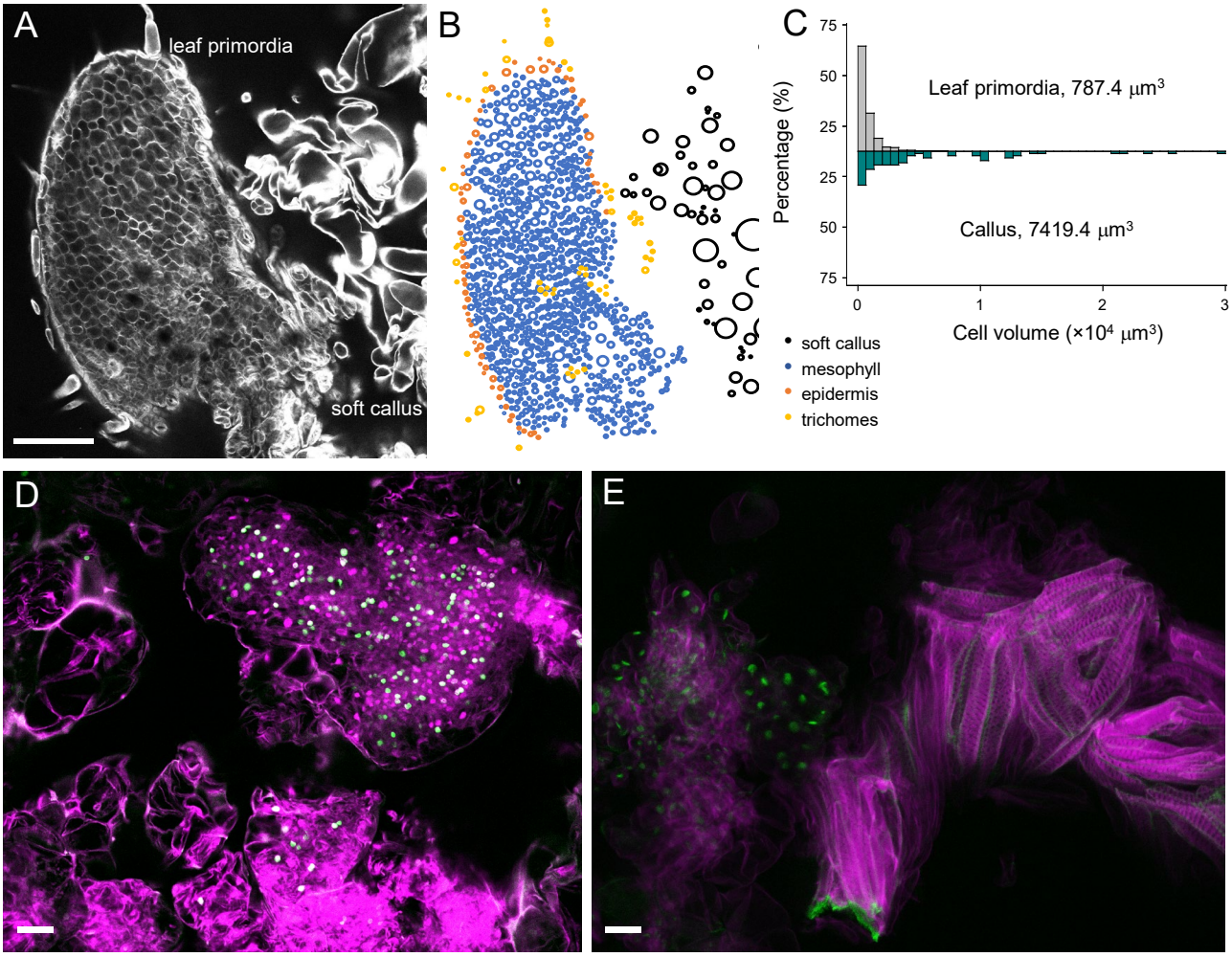
Suppl. Fig. S1. Hormone-induced shoot formation in M82 and MT tomato cultivars (cont.). Representative images of shoot formation in M82 (A, B) and MT (C) tomato cotyledon explants. Scale bars: 3 mm.



Suppl. Fig. S2. Experimental design to determine the effects of genotype and explant region on hormone-induced callus and shoot formation. (A) Apical, central, and basal regions of the cotyledon used as explants. M82 is shown as a representative example of the genotypes studied. (B) Representative images of explant incubation in supplemented medium for the six genotypes studied in this work. Scale bars: 3 mm..



Suppl. Fig. S3. Timing of callus and shoot formation in individual tomato explants of MT (A) and MM (B).



Suppl. Fig. S4. Cellular analysis of de novo leaf formation in tomato cotyledon explants. (A) A representative image of a leaf primordium and adjacent callus tissue in M82 cotyledon explants at 3 weeks. (B) Graphical representation of individual cell coordinates within the leaf primordium and adjacent callus indicated in B; bubble size represents cell volume. (C) Histogram of cell sizes from B. (D) Detailed observation of a globular callus tissue (green, 12 h EdU; magenta, SR 2200 cell wall staining) where de novo formed xylem cells are clearly visible (E). Scale bars: 40 μm.

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Review

Recent Advances in Tomato Gene Editing

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Abstract: The use of gene-editing tools, such as zinc finger nucleases, TALEN, and CRISPR/Cas, allows for the modification of physiological, morphological, and other characteristics in a wide range of crops to mitigate the negative effects of stress caused by anthropogenic climate change or biotic stresses. Importantly, these tools have the potential to improve crop resilience and increase yields in response to challenging environmental conditions. This review provides an overview of gene-editing techniques used in plants, focusing on the cultivated tomatoes. Several dozen genes that have been successfully edited with the CRISPR/Cas system were selected for inclusion to illustrate the possibilities of this technology in improving fruit yield and quality, tolerance to pathogens, or responses to drought and soil salinity, among other factors. Examples are also given of how the domestication of wild species can be accelerated using CRISPR/Cas to generate new crops that are better adapted to the new climatic situation or suited to use in indoor agriculture.

Keywords: zinc finger nucleases; TALE nucleases; CRISPR/Cas; *Solanum lycopersicum*; tomato domestication; plant architecture; abiotic stress; pathogen resistance

1. Introduction

Current global agricultural production faces significant losses due to increased environmental stress caused by climate change. This stress results from the fluctuations in temperature and precipitation, along with related factors such as soil salinization [1,2]. Consequently, the development of new tools to alleviate the negative effects of the current climate change scenario is crucial. In one avenue of development, to effectively respond to the various stresses caused by anthropogenic climate change, crops are currently undergoing genetic modification through new genomic techniques (NGTs) to enhance their resilience and adaptability [3]. Gene-editing tools, such as zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated proteins (Cas), are the most commonly used NGTs for crop improvement.

Tomatoes (*Solanum lycopersicum* L.) are the most widely grown vegetable crop, with 186.1 million metric tons produced worldwide in 2022 [4]. However, drought, increasing global temperatures, the spread of insect pests, bacteria, and viruses pose a threat to their current production [5]. To overcome this, gene-editing tools have increased the tolerance of tomato plants to adverse factors, including heat, drought, salinity, bacteria, and viruses. Furthermore, tomato plants with increased levels of lycopene and carotenoids, along with other biochemical enhancements, have been effectively produced using gene-editing tools. Against this background, in this review, we present an overview of the gene-editing tools that are available and the strategies used to obtain edited plants, the results attained in tomatoes when using these tools, and a discussion of the weaknesses and strengths of the current approaches.

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2. Gene-Editing Systems in Plants

2.1. Zinc Finger Nucleases (ZFNs)

Cys2-His2 (C2H2) zinc finger domains are present in many eukaryotic transcription factors. The classical C2H2 domain, with 28–30 amino acids, comprises two β-strands and an α-helix stabilized by a Zn^{2+} atom through conserved cysteine and histidine residues. Each C2H2 domain binds 3–4 nucleotides of DNA. Furthermore, the fusion of tandem C2H2 domains, separated by a conserved sequence of five amino acids, enables the binding to specific, longer DNA sequences. The zinc finger transcription factors were discovered in 1985 [6] and have since been utilized to regulate gene expression. ZFNs are chimeric proteins that comprise multiple C2H2 zinc finger domains, up to three, which are fused to the C-terminal cleavage domain of the endonuclease FokI, rendering ZFNs pioneering gene-editing tools to have been made accessible. The DNA sequence specificity of composite arrays of C2H2 zinc finger domains utilized to construct ZFNs determines the specificity of endonuclease cleavage. Dimerization of two different ZFN proteins results in a double strand break (DSB) in DNA with 5–7 nucleotide 5′ overhangs, as the Fok1 cleavage domain needs dimerization to cleave DNA (Figure 1a) [7,8]. The resulting DSBs in the DNA initiate endogenous mechanisms of DNA repair, predominantly through the non-homologous end-joining (NHEJ) pathway, which leads to the appearance of deletions and insertions at the repair site [9].

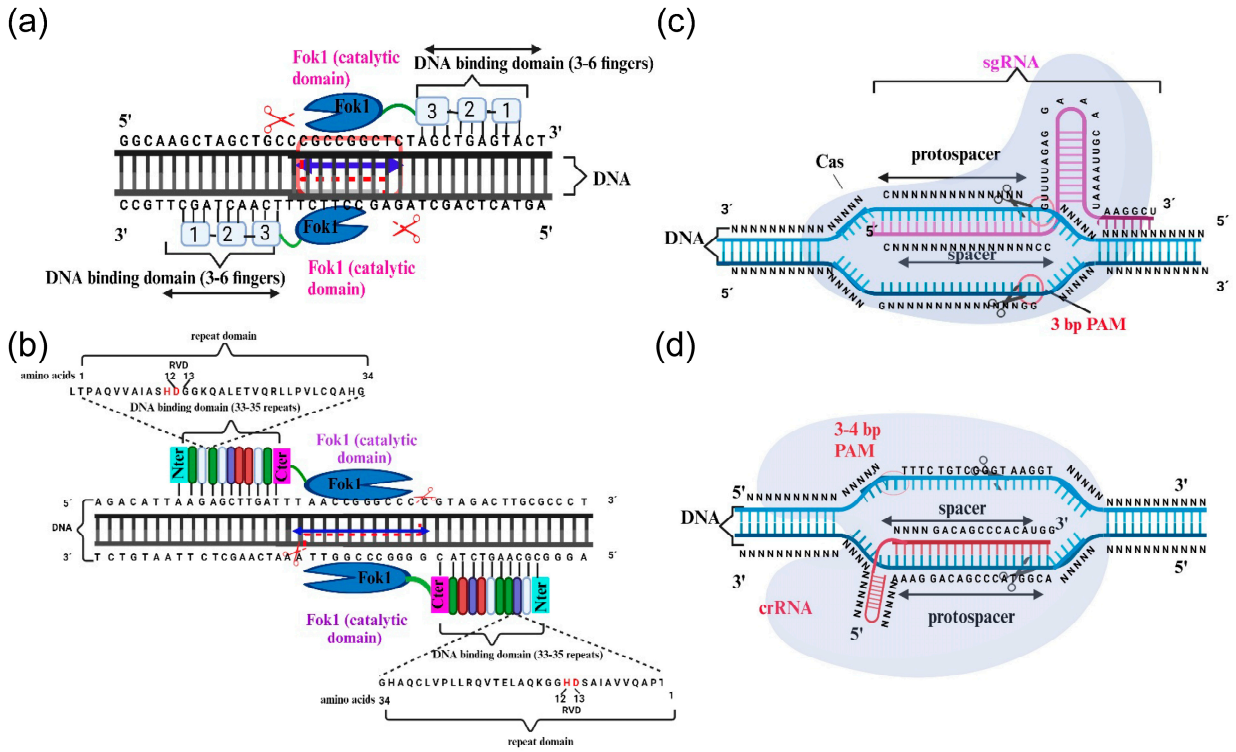

Figure 1. Overview of the gene-editing systems analyzed in this review. (**a**) ZFNs, (**b**) TALENs, (**c**) CRISPR/Cas9, and (**d**) CRISPR/Cas12a. The main text provides a thorough description of all the elements depicted in the different panels. Created with BioRender.com (accessed on 12 January 2024).

The editing efficiency of the ZFN system is generally poor, with a success rate ranging from only 1% to 10% [9–13]. This limitation could be due to potential interactions between the neighboring C2H2 domains in the ZFNs, which affect their DNA binding specificity, although the underlying mechanism remains unclear [14]. Due to their low specificity, ZFNs can cause unintended mutations at off-target sites, as evidenced by earlier studies [8]. The first plant to be edited using the ZFN system was the model plant *Arabidopsis thaliana* [15], followed by maize [9], tobacco [11,16,17], and soybean [18]; however, the scope of crop editing using ZFNs has been limited due to the potential drawbacks of this approach [19–21].

2.2. Transcriptional Activator-like Effector Nucleases (TALENs)

TALENs are also chimeric proteins that can be engineered to cleave particular DNA sequences. Similar to ZFNs, TALENs comprises the C-terminal domain of FokI endonuclease and specific DNA binding domains, originating from the transcription activator-like effectors (TALE) from plant-pathogenic bacteria [22]. The TALE domain consists of numerous (between 12 and 27) consecutive repeats of 33–35 amino acids, each containing two α-helices and a short repeat variable diresidue known as RVD. RVD is essential for making sequence-specific DNA contacts. By using both bioinformatics tools and empirical data, the association between the RVD amino acid sequence and its binding to a specific nucleotide in the DNA was established [23,24]. Shortly afterwards, newly engineered *TALE* genes were utilized to selectively upregulate endogenous genes in various plant species [25]. The assembly of repeat modules for different RVD arrays allows for the generation of sequence-specific DNA binding domains to whatever length is desired. Like ZFNs, two monomeric TALENs are necessary to bind the target sites in the DNA and enable FokI to dimerize and cut the DNA (Figure 1b). *Arabidopsis thaliana* [26] and rice [27] were the first plant species to be edited using TALENs. To date, over 50 genes in 12 plant species, such as maize, wheat, barley, tobacco, soybean, potato, and tomato, among others, have undergone successful gene editing through the use of TALENs [28].

When considered in comparison to ZFNs, the interactions between DNA-binding domains of TALENs and their target nucleotides are simpler than those between ZFNs and their target trinucleotides. This means TALEN design is generally less complex than that of ZFNs. Additionally, TALENs offer other benefits over ZFNs, including higher editing efficiency (approximately 30%), and lower off-target mutations [28]. However, a clear disadvantage of TALENs is their significantly larger size when compared with ZFNs, which can pose challenges for their delivery and expression.

2.3. Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and CRISPR-Associated Protein (Cas)

CRISPR and its associated locus encoding Cas proteins were initially discovered in the genomes of bacteria [29] and archaea [30]. The CRISPR/Cas system plays a crucial role in the adaptive immune responses against lytic bacteriophages and plasmids [31–33]. Upon initial infection by a phage, fragments of its DNA integrate into the CRISPR genomic locus. Upon new viral infection, the CRISPR locus is transcribed into the CRISPR RNA (crRNA), consisting of spacers with distinct sequences from various phages, indicative of past infections. These spacers are separated by repetitive endogenous sequences, known as direct repeats [34]. The Cas proteins possess nuclease and helicase domains that facilitate identifying and cutting exogenous DNA by following a crRNA-guided mechanism. Further additional processing requires trans-activating CRISPR RNA (tracrRNA) [35]. The tracrRNA–crRNA–Cas ribonuclease complex can recognize and cleave any exogenous DNA molecule that shares homology with the crRNA (Figure 1c). Another key element necessary for Cas cleavage activity is the so-called protospacer-adjacent motif (PAM) sequence, a conserved sequence of 2–6 base pairs flanking the target DNA sequence. Consequently, a sequence with perfect complementarity to the crRNA guide but lacking a PAM will be ignored by the Cas nuclease [36].

Soon after the molecular mechanism was uncovered, several research groups were able to edit genes in different organisms using the CRISPR/Cas system with great success [37–41]. A major step forward was the creation of a chimeric molecule called single guide RNA (sgRNA), which is a fusion of crRNA and tracrRNA and contains a short sequence complementary to the endogenous sequence of the gene to be edited. By varying the sgRNA sequence, it becomes possible to target different regions of DNA. Different CRISPR/Cas systems have been thoroughly investigated [42]. Two major classes, namely class 1 and class 2, comprise multi-subunit effector complexes or single-protein effector complexes, respectively, which are further divided into types (I, III, IV and II, V, VI, respectively) and subtypes [42,43]. Of the various Cas proteins identified, the most frequently used for gene

editing are type II-A Cas9 from *Streptococcus pyogenes* [42] and type V-A Cas12a (Cpf1) from *Acidaminococcus* sp. And *Lachnospiraceae bacterium* [44].

2.3.1. Cas9

The most common method of gene editing utilizes the Cas9 protein of *Streptococcus pyogenes*. The Cas9 protein is a dual RNA-guided DNA endonuclease able to cleave the target DNA in both strands through recognizing the hairpin loop architecture of the crRNA–tracrRNA complex [45]. Cas9 guided by multiple sgRNAs can bind to endogenous genes with high specificity. In 2013, the first CRISPR/Cas9 edited plants of *Arabidopsis thaliana*, *Nicotiana benthamiana*, and *Oryza sativa* were obtained [46,47]. Different versions of Cas9 with high efficiency for gene editing have been identified and utilized in plants since then [48].

When compared to other gene-editing approaches, the CRISPR/Cas9 system exhibits high rates of editing efficiency, approximately 75% to 85% [49–52], surpassing those of ZFNs and TALENs [53]. However, the CRISPR/Cas9 system for gene editing has several drawbacks, including reduced editing efficiency due to PAM sequence mismatches, genetic mosaicism, and editing of non-targeted regions of the genome (i.e., off-target gene editing). To address these issues, a mutant version of Cas9 called Cas9 nickase (nCas9) has been engineered to generate single-strand DNA breaks and reduce off-target effects [54].

2.3.2. Cas12

Generally, Cas12a and their orthologs are smaller than Cas9 as they possess the nuclease domain but lack the helicase domain (Figure 1d). Certain Cas12a proteins serve as single effectors, which induce DSBs on the target DNA and are guided by smaller crRNAs (~42 nt) in contrast to those of Cas9 crRNAs (~100 nt) [44,55]. Cas12a has the ability to process pre-crRNA into mature crRNA independently of the tracrRNA. Additionally, there are other unique features of Cas12a proteins that distinguish them from Cas9. These include their preference for thymidine-rich PAM, whereas Cas9 prefers guanidine-rich sequences. Furthermore, after Cas12a cleaves the target DNA, it produces a 5′ overhang, increasing the efficiency of precise gene editing in AT-rich target regions, which Cas9 has difficulty accessing [44]. Beyond that, while Cas9 and Cas12a exhibit similar mismatches in their target DNA when assessed in vitro [56], it has been observed that Cas12a displays a lower off-target effect than Cas9 when conducting gene editing [57,58]. Initial successful outcomes following gene editing in plants using Cpf1, which is the Cas12a ortholog from *Francisella novicida*, were achieved in 2016 for *Oryza sativa* and *Nicotiana benthamiana* [59].

2.3.3. Other Cas Proteins

Cas13a is an RNA-guided RNA endonuclease from the bacterium *Leptotrichia shahii* that does not cleave DNA, but only single-stranded RNA [60]. Its use has been proposed to engineer resistance against plant RNA viruses and regulate gene expression post-transcriptionally [61]. Additionally, a few recent studies demonstrated that the hypercompact CRISPR/Cas gene-editing system of Cas12j encoded in some phage genomes can be used for efficient gene editing in plants with expanded target recognition capabilities compared to other Cas proteins [62–64].

2.4. Other Gene-Editing Tools

Several other gene-editing tools have been developed utilizing Cas9's ability to bind to specific DNA sequences via their sgRNAs. These methods all employ non-functional variants of the Cas9 nuclease, also referred to as dead Cas9 (dCas9). dCas9 binds to the sgRNA target site on the DNA but does not cause DSBs, instead interfering with downstream transcription [65]. The fusion of specific deaminases with dCas9 enables the engineering of base editors, which direct the introduction of point mutations in specific sequences of a target DNA without initiating DSBs [66]. There are two classes of DNA base editors, namely cytosine base editors (CBEs) and adenine base editors (ABEs). ABEs convert

the A=T base pair into G≡C, whereas CBEs convert G≡C into the A=T base pair [48,67]. Base editing has been successfully applied to a range of agronomic traits in crop plants, including wheat, rice, and tomatoes [68,69]. In prime editing, the dCas9 is fused with a reverse transcriptase (RT) enzyme, allowing for the introduction of base changes at the targeted site via the RT activity and using a prime editing guide RNA (pegRNA). This pegRNA not only specifies the target site but also encodes the desired edit [70]. Efficient cases of prime editing were previously reported for monocots, including rice, wheat, and maize, followed by mosses and dicots [71].

The different gene-editing methods described in this section allow for the precise modification of specific regions of the plant genome. CRISPR/Cas systems are the most widely used gene-editing tool due to their simple design, low cost, high efficiency, good repeatability, and short cycle time. Nevertheless, it is important to consider the advantages and disadvantages of ZFNs, TALENs, and CRISPR/Cas systems for gene editing to improve crop performance (Table 1).

Table 1. Advantages and disadvantages of gene-editing systems in plants.

Editing System	Advantages	Disadvantages
ZFNs	First editing tool made available.	Low editing efficiency. High rates of off-target mutations. Assembling the ZFN array is time-consuming and requires a high level of expertise. Sensitive to DNA methylation. Not suitable for gene target multiplexing. Sequence length limitations in the target sequence.
TALENs	Targets any DNA sequence. Fewer off-target mutations. No length limitations in the target sequence.	Sensitive towards DNA methylation. Expensive and time-consuming design. Not appropriate for targeting multiple genes simultaneously.
CRISPR/Cas	Higher editing efficiency. Easier to design and relatively cheaper. Possibility of gene target multiplexing. Cas proteins work across different species. Low rates of off-target effects or no off-target effects if the sgRNA is optimized.	The choice of target gene is limited by the PAM motif.

3. Methods for Obtaining Gene-Edited Plants

3.1. Methods for CRISPR/Cas Delivery

In order to induce DSBs and activate the endogenous DNA repair mechanism, which ultimately leads to gene editing, the Cas protein and sgRNA(s) components of the CRISPR/Cas system must reach the nucleus of the plant cell. This can be achieved through conventional delivery of genetically encoded CRISPR/Cas components or through preassembled gRNA/Cas ribonucleoprotein (RNP) complexes. The RNP complexes target and cleave the target sites in the DNA immediately after delivery and are rapidly degraded in those cells. Several recent systematic reviews have described the different methods used to deliver the CRISPR/Cas system to plants [72–75], and we will not provide an exhaustive description of them here. Below, you will find a brief overview of these methods, with an emphasis on recent developments for CRISPR/Cas delivery in tomatoes.

3.1.1. Particle Bombardment

The biolistic method delivers molecules into plant nuclei using accelerated nano-sized particles coated with nucleic acids or RNP complexes (Figure 2a) [76]. However, there are several disadvantages to this approach, including the random integration of the cargo DNA at multiple sites in the genome and the high cost of the equipment and reagents. Regenerating whole plantlets from transformed explants is a time-consuming process that is also dependent on the species and the genotype. RNP-mediated gene editing using

particle bombardment has been successfully achieved in several species, but it has rarely been reported in tomatoes [77].

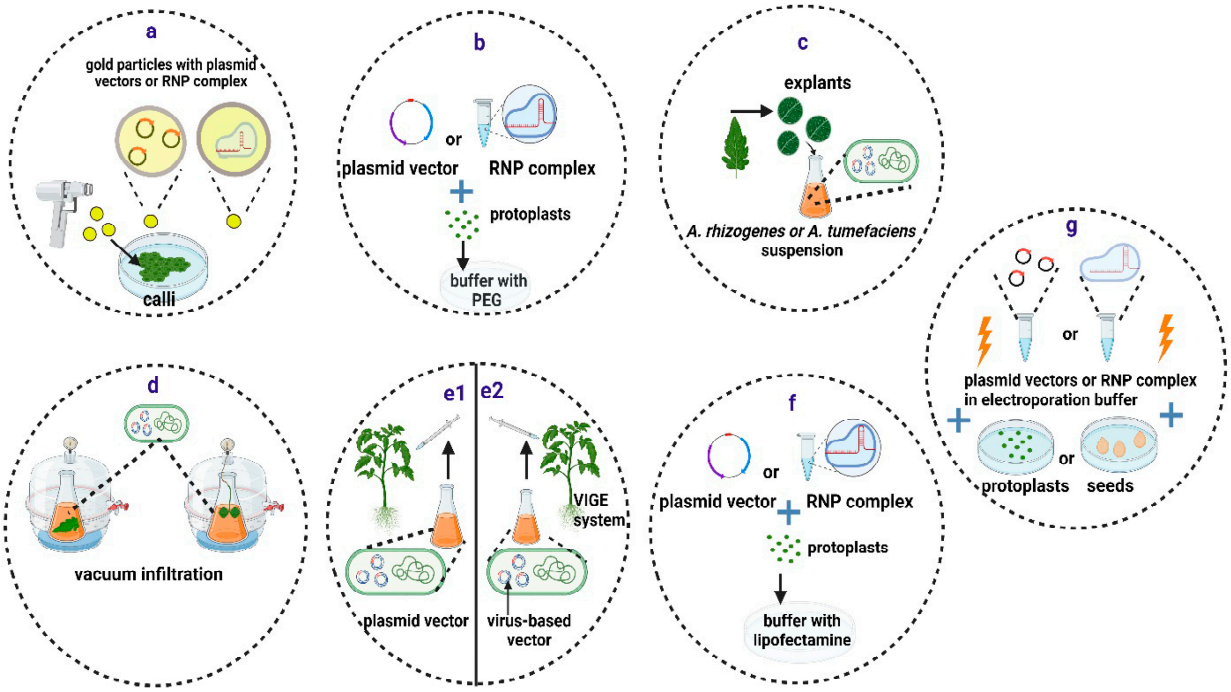

Figure 2. Methods for delivery of CRISPR/Cas components into plant cells and tissues. (**a**) Particle bombardment. (**b**) PEG-mediated transfection. (**c**–**e**) Biological methods, including Agrobacterium-mediated stable transformation (**c**), vacuum infiltration (**d**), and direct injection of the Agrobacterium culture with an integrative plasmid vector (**e1**) or a virus-based plasmid vector (**e2**). (**f**,**g**) Other methods, including liposome-mediated transfection (**f**) and electroporation (**g**). Created with BioRender.com (accessed on 12 January 2024).

3.1.2. Polyethylene Glycol (PEG)-Mediated Transfection

In the presence of divalent cations, such as Ca^{2+} or Mg^{2+}, and at high pH, PEG facilitates the incorporation of exogenous DNA or RNP complexes into plant protoplasts, but with low transformation efficiency (Figure 2b). This approach relies on the establishment of successful protoplast isolation and regeneration procedures. PEG-mediated transfection has been widely used to deliver vector DNA or RNPs into protoplasts of many plant species, including various tomato cultivars [78–81] and the wild tomato *S. peruvianum* [82].

3.1.3. Biological Methods

Biological methods for delivering DNA into plant nuclei rely on the natural ability of plant-pathogenic soil bacteria of the genus *Rhizobium* to integrate some of the genes present in their T-DNA into the genomic DNA of plant cells [83,84]. *A. tumefaciens*, also known as *Rhizobium radiobacter*, is the most commonly used species for delivering vectors encoding the CRISPR/Cas components. Conversely, *A. rhizogenes*, also known as *R. rhizogenes*, has received considerable attention in recent years during studies of root-specific processes [85]. A large number of laboratory strains of *A. tumefaciens* are available to transform a wide variety of plant species with varying success [86]. Recently, the *A. tumefaciens* and *A. rhizogenes* genomes were modified to improve their transformation efficiency and broaden their host range of plant species [87,88].

Agrobacterium spp. cell cultures containing the desired vectors in the exponential growth phase are used to transform plant tissues (Figure 2c). For plant species such as *A. thaliana*, the floral dip method [89] is preferred, while in most other cases, *A. tumefaciens* must be co-cultured with tissue explants, such as cotyledons, leaves, calli, or embryos. In

such cases, a suitable protocol for regenerating whole plants from the few transformed cells with the T-DNA from *A. tumefaciens* is required. In the case of tomatoes, cotyledons can be easily transformed through co-culturing with various *Agrobacterium* spp. strains. Shoot induction can then be produced shortly afterwards with appropriate hormone combinations [90].

Transient expression of CRISPR/Cas components can be induced in plant tissues by delivering *A. tumefaciens* into plant cells through direct injection or vacuum infiltration (Figure 2d,e1) [91]. This method is appropriate for evaluating the gene-editing efficiency of different vectors in tomato leaves before conducting stable transformations [92,93]. Zhang and coauthors (2020) tested 195 sgRNAs for their ability to cause mutations in tomato leaves, and they found that 61.5% of them were suitable for gene editing [92].

Virus-induced gene editing (VIGE) relies on the use of viral-derived vector systems for the delivery of CRISPR/Cas components into plant cells [94]. The VIGE approach does not require genome integration of the CRISPR/Cas backbone, making it less time-consuming and more broadly applicable. However, the primary limitation of this approach is that most viruses are unable to infect meristematic or germline cells. As a result, regenerated edited plants are normally derived from other somatic cells [94]. Nonetheless, various approaches based on vectors derived from geminivirus [95] and potato virus X [96] have been successfully employed to produce stable edited tomato plants using direct injection (Figure 2e2). The results suggest that the VIGE approach is a reliable and adaptable technology that can be used for precise breeding of tomato traits.

3.1.4. Other Methods of CRISPR/Cas Delivery

Liposomes are lipid-based nanoparticles that have been shown to offer a useful tool for the delivery of genes and proteins (Figure 2f) into mammalian cells [97] and that have been used to deliver donor DNA and support subsequent successful gene editing in citrus protoplasts [98]. In addition, RNPs have been delivered in tobacco protoplasts through liposome nanoparticles, achieving editing efficiencies as high as 6.0% [99]. Moreover, recently, a similar protocol has been devised for the successful delivery of RNPs in maize protoplasts [100].

Electroporation, meanwhile, is a technique that increases the permeability of cell membranes in plant cells without cell walls (protoplasts) by exposing them to short and intense electric pulses (Figure 2g). This allows for the incorporation of exogenous nucleic acids or RNPs [101]. Recently, researchers have demonstrated the delivery of RNPs into cabbage and soybean protoplasts via electroporation, achieving editing efficiencies as high as 3.4% and 8.1%, respectively [102,103]. Electroporation has also been used in tomatoes for the delivery and transient expression of vectors encoding ZFNs [104], but no other reports with CRISPR/Cas vectors of RNPs can be found in this species so far.

3.2. Marker Genes Used with the CRISPR/Cas System

When using conventional CRISPR/Cas vectors, most gene-edited plants include the stable integration of the CRISPR/Cas system into the plant genome. Furthermore, the CRISPR/Cas expression module typically includes genes encoding selection markers for antibiotic or herbicide resistance. However, the presence of the transgene does not necessarily indicate that gene editing has taken place. Characterizing the mutations that have occurred in the target DNA often requires laborious procedures, including high-resolution melting PCR or Sanger sequencing. Additionally, to conform with biosafety regulations concerning genetically modified organisms (GMOs), the CRISPR/Cas expression module must be segregated in the progeny of gene-edited plants through self-pollination or crossing. Alternatively, the delivery of preassembled CRISPR/Cas components is considered a transgene-free method of gene editing. In this approach, the RNP complexes have high specificity for their target DNAs and are rapidly degraded within the cell after dissociating from the genomic DNA. The fusion of Cas proteins with fluorescent proteins, such as

enhanced GFP (EGFP), enables the direct visualization of the RNP complexes in plant nuclei, which is highly convenient during the optimization of the delivery process.

For both approaches, it is beneficial to select a CRISPR/Cas-induced mutation that causes a visible phenotype. A frequently used endogenous target gene is the *PHYTOENE DESATURASE* (*PDS*) gene *Solyc03g123760*, which is involved in carotenoid biosynthesis. Mutations in this gene impair chlorophyll accumulation and produce an albino phenotype in many species, including tomatoes [105]. However, mutations in *PDS* affect the survival of the edited explants, which hinders the effective plant regeneration required for successive characterization. Recently, Rinne et al. (2021) proposed using a different endogenous marker gene to evaluate CRISPR/Cas gene-editing activity [106]. The *MULTIPLE ANTIBIOTIC RESISTANCE 1* (*MAR1*) gene *Solyc01g100610* encodes a transporter located in the mitochondria and chloroplasts, which plays a role in maintaining iron homeostasis and facilitates the transport of aminoglycoside antibiotics into these organelles. Mutations induced by the CRISPR/Cas system in *MAR1* confer kanamycin resistance in tomatoes [106].

4. Gene Editing in Tomato Breeding

4.1. ZFNs

Only a limited number of studies are available on the application of ZFNs to edit tomato plants [107,108]. Shukla et al. (2016) conducted an experiment where they utilized ZFNs to target *Solyc07g062650*, which encodes a mitochondrial malate dehydrogenase (mMDH), in both the M82 and Moneymaker tomato cultivars [107]. PEG-mediated transfection was used to deliver four distinct ZFN constructs into protoplasts, and the regenerated plantlets were obtained via indirect organogenesis. The editing efficiency of *mMDH* was found to be low, ranging from 0.7% to 5.5%. The majority of mutants were found to have small deletions varying from 1 to 22 bp, while one mutant showed an insertion of 2 bp. Regenerated mutants displayed different phenotypes: heterozygous plants for any mutation that led to disruption of the *mMDH* open reading frame showed a decrease in growth and fruit yield, while homozygous plants for specific mutations exhibited an increase in fruit yield [107]. In another study, ZFNs were used to target the *LEAFY-COTYLEDON1-LIKE4* (*L1L4*) gene *Solyc05g005350*, which encodes for the β-subunit of a nuclear factor Y, in the Heinz 1706 cultivar [108]. Electroporation of germinated seeds facilitated the transient expression of constructs encoding a pair of ZFNs designed to target exons 1 and 2 of the *L1L4* gene. Based on the phenotypes observed in the edited plants from the T_0 generation, over 65% efficiency was reported. Several mutations in the coding sequence of *L1L4* have led to variations in plant traits, including seedling vigor, plant height, number of florets, and flowering and ripening times, thereby demonstrating that the affected *L1L4* gene is a useful target for crop improvement [108].

4.2. TALENs

Three reports have been published to date on the use of TALENs for tomato gene editing. Lor et al. (2014) designed two pair of TALENs, under the control of an estrogen-inducible promoter, to target the *PROCERA* (*PRO*) gene in tomatoes, *Solyc11g011260* [109]. This gene encodes a negative regulator of gibberellin signaling. Upon TALEN expression, 7 out of 40 regenerated plantlets carried deletions ranging from 1 to 88 bp, along with a 39-bp insertion and a 4-bp deletion, resulting in frameshifts that would lead to the production of truncated PRO proteins. Homozygous *pro* plants displayed phenotypes indicating an increased gibberellin response [109]. In another report, geminivirus replicons were used to express TALENs designed to target the *ANTHOCYANIN 1* (*ANT1*) gene *Solyc10g086260*, encoding an MYB transcription factor whose overexpression results in purple plant tissue due to anthocyanin accumulation. The gene-editing efficiency of this system was about 14% [95]. In a recent report, a known mutation in the pepper gene that encodes *EUKARYOTIC TRANSLATION INITIATION FACTOR 4E* (*eIF4E*), *Solyc03g005870*, which is reported to be associated with potyvirus resistance, was generated through a gene

knock-in strategy with a pair of TALENs in tomato [110]. The TALEN vector and the donor DNA template were introduced to cherry tomato leaves in a biolistic approach. One out of thirty-two regenerating shoots incorporated the donor template through NHEJ, and the edited plants with this allele showed the broadest potyvirus resistance spectrum achieved through genetic engineering in tomatoes so far [110].

4.3. CRISPR/Cas9

The CRISPR/Cas9 approach was first applied to tomato gene editing in 2014 [111,112]. As a proof of principle, Brooks et al. (2014) designed two sgRNAs to target the tomato homolog of the *ARGONAUTE7* (*AGO7*) gene *Solyc01g010970* [111], since loss-of-function mutations of *AGO7* are known to produce filiform leaves [113]. The plasmid vector was delivered via *Agrobacterium tumefaciens* transformation of M82 cotyledons, and 14 out of 29 T_0 plants displayed the wiry leaf phenotype characteristic of known *ago7* alleles [111]. Ron et al. (2014), meanwhile, used *A. rhizogenes*-mediated transformation of cotyledon explants as a delivery method for the CRISPR/Cas9 vector, which was designed to target both a reporter gene (*mGFP5*) and the *Solyc02g092370* gene encoding a *SHORT-ROOT* (*SHR*) protein in tomato hairy roots [112]. Consistent with the notion of a conserved role of SHR in both tomatoes and *A. thaliana*, transgenic hairy roots with no GFP expression and a reduced root meristem were identified. These roots contained small insertions and deletions in the coding region of the *SHR* gene [112].

In the past decade, the use of CRISPR/Cas9 for gene editing in tomatoes has been implemented in dozens of tomato cultivars [114,115], including wild species such as *S. lycopersicum* var. *cerasiforme* [110], *S. pimpinellifolium* [116–121], and *S. peruvianum* [82]. When searching the scientific literature available as of 31 December 2023, we identified 356 primary references that used CRISPR/Cas technology for gene editing in tomato and related species (Figure 3a–c and Supplementary Table S1). From this list, we selected a subset of references to be further discussed (Supplementary Table S2). A Sankey diagram constructed from the evidence presented in these selected references illustrates the relationship between tomato cultivars, their wild relatives, Cas enzyme types, *A. tumefaciens* strains, and the key genetic traits influenced by the genetic modifications induced by CRISPR/Cas9 (Figure 3d).

Several studies have documented the domestication process from the most likely progenitor, *S. pimpinellifolium*, to modern tomato varieties [122]. Loss-of-function mutations in six loci that are important for key domestication traits have been identified from these and other studies: plant architecture (*SELF PRUNING* [*SP*] *Solyc06g074350*), fruit shape (*OVATE* [*O*] *Solyc02g085500*), fruit size (*FASCIATED* [*FAS*] *Solyc11g071380*, and *FRUIT WEIGHT 2.2* [*FW2.2*] *Solyc02g090730*), fruit number (*MULTIFLORA* [*MULT*] *Solyc02g077390*), and nutritional quality (*LYCOPENE BETA CYCLASE* [*CycB*] *Solyc04g040190*). To reconstruct the domestication of tomatoes from *S. pimpinellifolium*, Zsögon et al. (2018) [121] designed a CRISPR/Cas9 vector containing six sgRNAs that targeted specific sequences in the coding regions of these six genes. Four of the six targeted loci were successfully edited in all 50 T_1 lines tested, resulting in indel mutations in *SP*, *O*, *FW2.2*, and *CycB* [121]. In a subsequent round of gene editing, the researchers incorporated *CLAVATA 3* (*CLV3*) *Solyc11g071380*, and developed a new CRISPR/Cas9 vector with eight sgRNAs targeting *CLV3*, *FW2.2*, *MULT*, and *CycB* genes. This resulted in 28 T_1 lines with loss-of-function mutations in all four targeted loci [121]. The engineered lines exhibit a threefold increase in fruit size and a tenfold increase in fruit number when compared with the wild parent, *S. pimpinellifolium*. A significant finding was that the accumulation of fruit lycopene in these lines was enhanced fivefold in comparison to the cultivated tomato *S. lycopersicum* [121]. Similarly, Li et al. (2018) developed a multiplex CRISPR/Cas9 strategy to edit five genes in *S. pimpinellifolium* related to day-length sensitivity (*SELF PRUNING 5G* [*SP5G*] *Solyc05g053850*), shoot architecture (*SP*), flower and fruit production (*CLV3* and *WUSCHEL* [*WUS*] *Solyc02g083950*), and vitamin content (*GGP1 Solyc02g091510*) [119]. Furthermore, Lemmon et al. (2018) used CRISPR/Cas9 to mutate orthologs of tomato domestication genes that control plant architecture (*SP*), flower production (*SP5G*), fruit size (*CLAVATA1*), and fruit abscission

(*JOINTLESS-2*) in the orphan crop *Physalis pruinosa* [123]. This wild *Solanaceae* is distantly related to tomato and is grown in Central and South America for its subtly sweet berries. Overall, the results suggested that CRISPR/Cas9-mediated gene editing could be used to accelerate domestication in wild species and create crops that are better suited to changing climate scenarios [124,125].

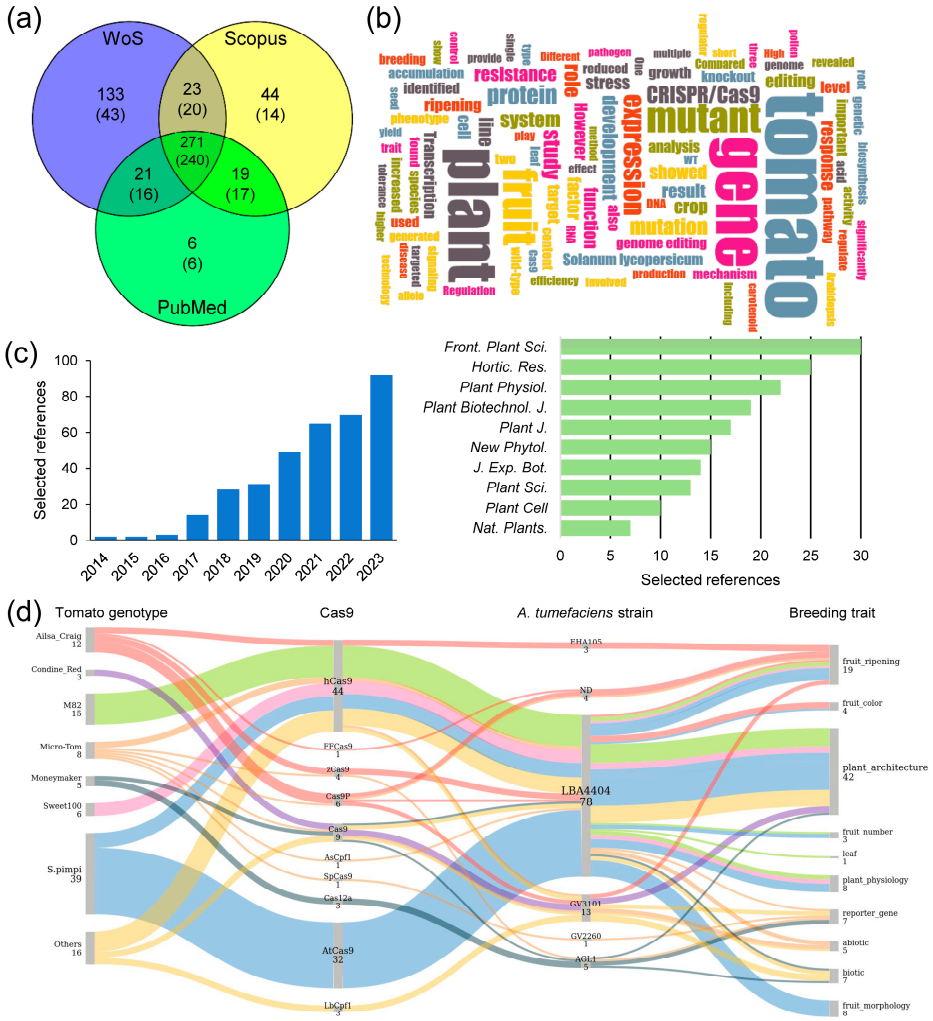

Figure 3. Results of a reference search and text mining analysis. (**a**) Venn diagram of scientific articles downloaded from public databases using the search term: "(Cas9 tomato) NOT (review)" and restricted by date (from 1 January 2014 to 31 December 2023). Numbers in brackets indicate selected references after manual curation. (**b**) A word cloud generated with the 356 unique abstracts selected based on the criterion of including any mutant generated by gene editing in tomato and related species in the main text. The image was generated using the web application located at https://wordclouds.ethz.ch/ (accessed on 12 January 2024). (**c**) Selected papers about gene editing in tomato and related species, along with the top 10 plant science journals in which they were published. The raw data utilized for preparing panels a–c can be found in Supplementary Table S1. (**d**) The Sankey diagram illustrates the relationship between tomato cultivars, types of Cas enzyme, *A. tumefaciens* strains, and the key breeding traits it influences. The diagram was generated with SankeyMATIC and is based on data from Supplementary Table S2. The thickness of the lines and the colors represents the proportion of evidence connecting the different categories. Spimpi: *S. pimpinellifolium* genotypes; ND: not included.

4.3.1. CRISPR/Cas9-Edited Genes Related to Fruit Characteristics

Tomato plants are grown for their fleshy berry fruits, which come in varying sizes, shapes, and colors [126,127]. Tomatoes contain several health-promoting components, including vitamins, carotenoids, and phenolic compounds [128]. Tomato breeders have long utilized the limited genetic variation present in this species to enhance fruit quality traits. Some of the genes responsible for variations in fruit weight, shape, and biochemical composition have been identified and those might be used for precise gene editing using the CRISPR/Cas9 system (Supplementary Table S2), as will be exemplified below.

Tomato fruit ripening is a complex physiological and biochemical process that involves the degradation of chlorophyll, the accumulation of pigments (mainly carotenoids), the softening of the pulp, and the alteration of its organoleptic qualities. This process is controlled by ethylene, and requires three transcription factors: the MADS-box RIPENING INHIBITOR (RIN), the SBP-box COLORLESS NON-RIPENING (CNR), and the NAC transcription factor NON-RIPENING (NOR) [129]. One of the initial reports on the application of CRISPR/Cas9 technology in tomatoes targeted the *RIN* gene (*Solyc05g012020*) [130,131]. Fruits from *RIN*-knockout plants exhibited partial ripening and lower levels of lycopene compared to the wild type [131]. However, unexpectedly, these fruits showed excessive cell wall degradation. Furthermore, CRISPR/Cas9-induced mutations in *CNR* (*Solyc02g077920*) and *NOR* (*Solyc10g006880*) did not abolish ripening [132,133], indicating that the ripening transcriptional regulatory network is highly robust. These results are in stark contrast with those found in "classical" *rin*, *Cnr*, and *nor* mutants, which have obvious ripening-inhibited phenotypes. The difference in findings may be explained by the recent proposal that some of these mutants have gain-of-function mutations [134].

The color change that occurs during fruit ripening is due to the accumulation of β-carotene (orange) and lycopene (red), as well as a decrease in β-xanthophyll (yellow) and chlorophyll (green) levels [135]. Furthermore, the expression of *SNAC4* (*Solyc07g063420*) and *SNAC9* (*Solyc04g005610*) genes is induced by ethylene, and alterations in the pigmentation of mature fruits are observed when the expression levels of these genes are reduced by VIGS, indicating a positive role of both genes in this process [136]. In previous research, the *SNAC9* gene was knocked out thoroughly using CRISPR/Cas9, resulting in an absence of expression of the protein. This led to a significant reduction in lycopene and total carotenoid contents in the mutants, possibly due to the direct regulation of key genes involved in carotenoid biosynthesis, such as *PHYTOENE SYNTHASE 1* (*PSY1*) *Solyc03g031860* [137]. A strategy using NGTs has recently been implemented to rapidly generate tomato lines with fruits of varying colors [135,138,139]. By genetically editing three genes related to fruit color, *PSY1*, *MYB12* (*Solyc01g079620*), and *STAY-GREEN 1* (*Solyc08g080090*), using a multiple CRISPR/Cas9 approach, it was possible to obtain transgene-free plants with fruits of different colors in less than a year. This strategy does not affect other important agronomic traits, such as yield and fruit quality [138].

Initial attempts to extend the shelf life of tomato fruits using CRISPR/Cas9 were focused on genes that encode enzymes responsible for degrading cell-wall pectins, such as pectate lyase (PL), polygalacturonase, and β-galactanase. However, only mutations in the *PL* gene *Solyc03g111690*, resulted in firmer fruits [140]. Simultaneous knockout of two genes, one encoding a fruit ripening-associated α-expansin (*Solyc06g051800*) and the other an endoglucanase (*Solyc09g010210*), using CRISPR/Cas9 improved fruit firmness [141]. Conversely, simultaneous overexpression of these genes promoted early fruit softening. These results support the conclusion that these two genes have a synergistic effect on fruit softening [141]. Furthermore, the bHLH transcription factor BRI1-EMS-SUPPRESSOR1 (BES1) encoded by *Solyc04g079980* has been identified as an upstream regulator of fruit softening through the activation of cell-wall degradation during ripening [142]. CRISPR/Cas9-generated loss-of-function *BES1* mutants resulted in firmer fruits and a longer shelf life during postharvest storage, without any negative impact on visual or nutritional quality.

4.3.2. CRISPR/Cas9-Edited Genes Related to Plant Architecture Traits

Modifying the plant architecture can significantly impact crop yield. This was demonstrated through mutations to the biosynthesis or signaling pathways of gibberellins, which increased crop yield by decreasing plant height in wheat and rice, an outcome that is of value during the "Green Revolution" [143]. Furthermore, in rice, the *IDEAL PLANT ARCHITECTURE 1* (*IPA1*) gene has been determined to be a novel master regulator of the plant architecture, which can be used as a target during molecular design to improve grain yield. *IPA1* encodes an SBP-box-like (SPL) transcription factor downstream of strigolactone signaling [144]. One of the *IPA1* orthologs of tomatoes, *SPL13 Solyc05g015840*, has been shown to act downstream of strigolactones to suppress lateral bud growth by inhibiting cytokinin biosynthesis. The knockout lines of *SPL13* generated with CRISPR/Cas9 displayed an increased shoot branching phenotype [133]. In other work, Kwon et al. (2020) developed a gene-editing staking approach to transform vine-like tomato plants into compact, early-yielding plants suitable for indoor agriculture [145,146]. Elsewhere, mutations in the classical flowering repressor gene *SP*, both natural and CRISPR/Cas9-induced, conferred a determinate growth habit in tomatoes [121]. Furthermore, mutating its paralog *SP5G* with CRISPR/Cas9 in the *sp* mutant background resulted in a shorter time to flowering and a more compact, determinate growth habit. This led to a quick burst of flower production and an early yield [147]. Meanwhile, cloning the *short internodes* dwarf mutant in the M82 cultivar was found to affect the well-known *ERECTA* (*ER*) gene [145]. In *A. thaliana*, this gene is known to regulate internode length [148]. The specific *ER* gene *Solyc08g061560* was targeted using CRISPR/Cas9 in the previously generated *sp sp5g* double mutant [147]. The resulting plants exhibited a triple-determinate form, and when grown in a self-contained, climate-controlled LED hydroponic vertical farm system, they produced higher yields due to their bushy shoot architecture, rapid cycling, and highly compact fruit clusters [145].

Naturally occurring mutations in the MADS-box transcription factor genes *JOINTLESS* (*J*) and *JOINTLESS2* (*J2*) in tomatoes have the potential to improve harvestability by modifying flower development, as they suppress the abscission zone in fruit peduncles [149]. The jointless pedicel trait has been successfully introgressed into small-fruited processed tomatoes and fresh-market tomatoes. However, the *j2* loss-of-function mutation can lead to undesirable branching of inflorescences in genetic backgrounds that also carry a cryptic variant for the close homolog *ENHANCER OF J2* (*EJ2*) *Solyc03g114840*, which was selected during domestication. This combination of *j2 ej2* loss-of-function alleles results in excessive flower production and low fertility due to a poor fruit set [150]. Deletions induced by CRISPR/Cas9 in *J2 Solyc12g038510*, result in the jointless phenotype. When combined with CRISPR/Cas9-induced null mutations in *EJ2*, this leads to strongly branched inflorescence in tomato breeding lines carrying the *suppressor of branching 3* (*sb3*) quantitative trait loci (QTL). These results suggest that genotypes carrying *sb3* could be utilized to maintain a normal inflorescence architecture when generating jointless phenotypes via gene editing [150].

During the process of tomato domestication, natural genetic variants were selected based on their alterations in the expression of key genes involved in target agronomic traits, such as yield or plant architecture. In many cases, the molecular characterization of these genetic variants has shown that they do not affect the coding region of the gene, but rather its *cis*-regulatory regions, either in gene promoters or in regulatory introns. Structural variations, such as large indels, duplications, and chromosomal rearrangements, play a crucial role in plant evolution and agriculture. They impact traits such as shoot architecture, flowering time, fruit size, and stress resistance [151]. Gene editing may be used to study the effects of variations in regulatory regions by recreating specific mutations or mimicking the expression effects of natural *cis*-regulators, which could be achieved by using the CRISPR/Cas approach on defined tomato genotypes. Accordingly, Rodríguez-Leal et al. (2017) developed a genetic screen that uses heterozygous loss-of-function mutant backgrounds to efficiently assess the phenotypic effects of multiple CRISPR/Cas9-induced promoter variants for known genes that regulate three key productivity traits in

tomato: fruit size (*CLV3*, *WUS*), inflorescence branching (*MULT*), and plant architecture (*SP*) [117]. The promoter alleles for *CLV3* exhibited a full range of quantitative variation. The edited plants displayed phenotypes ranging from moderate to strong, capturing the full spectrum of allelic diversity and locule number variation previously identified [117]. This approach has been demonstrated to generate multiple regulatory mutations for the systematic assessment of the association between *cis*-regulatory regions and phenotypic variation. Furthermore, there is also potential for engineering gain-of-function alleles and thereby establishing a basis for dissecting the complex relationships between gene-regulatory alterations, which may facilitate quantitative trait control.

4.3.3. CRISPR/Cas9-Edited Genes Related to Adaptive Stress Responses

Systematic reviews have compiled extant studies on gene editing with CRISPR/Cas9 in agricultural crops in the scientific literature [152–154]. Various biotic stresses, such as diseases and pests, as well as abiotic stresses such as cold, heat, drought, and salinity, affect tomato crop production, and Supplementary Table S2 shows that CRISPR/Cas9 gene editing has been used to address many of these traits. Those applications will be summarized in this section.

The *MILDEW RESISTANCE LOCUS O* (*MLO*) encodes an integral transmembrane protein that is highly conserved in monocots and dicots. It is a key factor in susceptibility to powdery mildew caused by the pathogenic fungus *Oidium neolycopersici*. In cultivated tomato and the wild species *S. peruvianum*, CRISPR/Cas9-induced knock-out lines of *MLO1* orthologs *Solyc04g049090* and *pSolyc04g049090* conferred resistance to *O. neolycopersici* without generating any other undesired phenotypic effects [82,155,156]. Furthermore, the *DOWNY MILDEW RESISTANT 6* (*DMR6*) gene in *A. thaliana* encodes a putative 2-oxoglutarate Fe(II)-dependent dioxygenase that has been identified as a factor in susceptibility to bacterial and oomycete pathogens. The tomato genome contains two orthologs of *DMR6*, *DMR6-1* (*Solyc03g080190*) and *DMR6-2* (*Solyc06g073080*). Inactivation of *DMR6-1* using CRISPR/Cas9 resulted in enhanced disease resistance against different tomato pathogens, such as bacteria, oomycetes, and fungi, without an obvious growth penalty [157]. Notably, the enhanced pathogen resistance observed in the *dmr6-1* tomato mutants was correlated with increased levels of salicylic acid. The biochemical characterization of DMR6 enzymes suggests that they play a role in converting salicylic acid to its inactive form [157]. In other work, the CRISPR/Cas9 system has been used to mutate the susceptibility gene *POWDERY MILDEW RESISTANCE 4* (*PMR4*) *Solyc07g053980*, with the product functioning as a callose synthase conferring resistance to *O. neolycopersici* and *Phytophtora infestans* in tomatoes [158,159]. These results demonstrate that the CRISPR/Cas9 system is suitable for facilitating broad resistance to bacterial and fungal pathogens by precisely targeting susceptibility genes and negative regulators involved in the plant defense mechanism.

Tomato yellow leaf curl virus (TYLCV) is a highly destructive viral pathogen that affects tomato crops globally. TYLCV is transmitted by the whitefly *Bemisia tabaci* when it feeds on the phloem sap of plants. Previous strategies to confer resistance to TYLCV in tomato plants have focused on the viral coat protein and DNA replicase protein, CP and RepA, respectively. Efficient viral interference was achieved by targeting the TYLCV genome with CRISPR/Cas9 for these sequences, resulting in reduced TYLCV accumulation in transgenic tomato plants [160]. Several QTL related to TYLCV resistance in tomatoes, including *Ty-1* to *Ty-6*, have been identified [161]. *ty-5* confers broad-spectrum resistance and encodes a missense allele of the tomato homolog of the PELOTA messenger RNA surveillance factor, which is involved in ribosome recycling during protein translation. Gene editing of the *PELOTA* gene *Solyc04g009810*, using a CRISPR/Cas9 approach resulted in resistance to TYLCV as a result of restricting the proliferation of viral DNA, likely by slowing or inhibiting ribosome recycling and decreasing viral protein synthesis in infected cells [156]. Targeting susceptibility factors encoded by the host plant genome, rather than

the viral genome, offers a promising approach to achieving resistance to pathogens without the need for stable inheritance of CRISPR/Cas9 components.

In the context of global warming, drought stress is becoming a critical challenge in tomato production. As previously discussed [162], the development of new CRISPR/Cas9-based approaches to improve drought tolerance in tomatoes is essential to reduce yield loss. Several key factors have been considered (Supplementary Table S2). Among those, in a scenario where the tomato *LATERAL ORGAN BOUNDARIES DOMAIN 40* (*LBD40*) *Solyc02g085910* was highly expressed in the roots and fruits and its expression was significantly induced by PEG and salt treatment, *LBD40* knockout mutants generated by CRISPR/Cas9 gene editing improved the water-holding ability of tomatoes under drought conditions, suggesting that *LBD40* was a negative regulator of drought tolerance in this species [163]. Furthermore, two studies have highlighted the role of *AUXIN RESPONSE FACTOR 4* (*ARF4*) *Solyc11g069190* in mediating the tolerance to salinity and osmotic stress in tomatoes [164,165]. The expression of the *ARF4* gene was downregulated in tomato seedlings under ABA and water deficit conditions. Downregulating the expression of *ARF4* with CRISPR/Cas9 was observed to enhance salt and osmotic stress tolerance recovery. This resulted in an obvious leaf curling phenotype, which reduced transpiration, and a significant increase in root length and density compared to wild type plants under stress conditions [164,165]. More recently, the expression of *SP2G* (*Solyc02g079290*) and *SP3C* (*Solyc03g026050*) was found to be significantly increased in the leaves of the drought-resistant wild species *S. pennellii* and in domesticated tomatoes after irrigation was stopped [166]. Furthermore, three independent CRISPR/Cas9-mediated *SP3C* homozygous mutants exhibited increased root length and reduced lateral root branching compared to the wild type. These traits are associated with greater drought tolerance in tomatoes and could be fine-tuned for agronomic gain [166]. Further research into the mechanisms of action of key transcription factors that regulate the abiotic stress response in tomatoes may expand the resources and tools at our disposal for developing multi-stress tolerant tomato varieties.

4.4. CRISPR/Cas12a

Two different Cas12a variants were used to stably transform tomato plants so they targeted *Solyc01g079620*, which encodes the MYB12 transcription factor required for flavonoid biosynthesis. A mutation in this gene leads to the production of pink-colored fruits [167]. The gene-editing efficiency of this system ranged from 7.7% to 48.8%, with a tendency to induce a broader range of deletions but no insertions, in contrast to Cas9 [168]. Furthermore, as demonstrated by Vu et al. (2020), the CRISPR/Cas12a system has the advantage of an increased efficiency in genome editing via the homology-directed repair (HDR) pathway when compared to Cas9. The authors reported an HDR efficiency of 4.5% of the *ANT1* visible marker when using a geminiviral replicon system [169]. In another study, to confirm the applicability of the Cas12a-mediated HDR approach for tomato genome editing of a potential agronomic trait, a known mutation in the *HIGH-AFFINITY* K^+ *TRANSPORTER 1;2* (*HKT1;2*) gene *Solyc07g014680*, which determines salinity tolerance [170], was engineered. However, the efficiency of HDR was low, at only 0.7%. The edited plants were salt-tolerant in both homozygous and heterozygous states [169]. In other work, a higher HDR efficiency of 4.3% was reported when using a new Cas12a variant that is capable of performing the cleavage of the target DNA at low temperatures [171]. Together, these results confirm that Cas12a-mediated HDR induces efficient gene targeting, which may be used to obtain allele-specific traits and marker-free tomato plants. More recently, Cas12 has been fused to a CBE with the aim of conferring resistance to the herbicide chlorsulfuron via precise editing of the *ACETOLACTATE SYNTHASE* (*ALS*) gene *Solyc03g044330* [172]. More than 20 chlorsulfuron-resistant lines were obtained in this way and the mutations were confirmed to be highly specific. In addition, the authors edited the *Solyc08g061560* gene, which encodes the ER kinase receptor. Mutations in this gene in *A. thaliana* resulted in a compact

inflorescence [148]. The phenotype of the homozygous edited tomato plants in this gene included a compact architecture, short petioles, and densely clustered inflorescence [172].

5. Conclusions

Over the last 15 years, significant progress has been made in crop gene editing using the NGTs of ZFNs, TALENs, and CRISPR/Cas. Among these, the CRISPR/Cas-based editing system has emerged as the preferred option due to its many benefits. The high efficiency of gene editing induced by the Cas proteins, together with the flexibility in the design of gene-specific RNA guides, allows the chosen experimental design to be refined while considering the genotype of the tomato variety, thus supporting the ultimate objective pursued with the genetic modification. A wealth of evidence supports the utility of the CRISPR/Cas system for the genetic editing of multiple genes in different varieties of cultivated tomatoes and related wild species. In this review, we conducted a systematic search of the references and identified 356 scientific articles that reported primary results on the modification of a gene using NGTs in cultivated tomatoes or related wild species. From these, we selected 47 genes that affect key genetic traits that we determine to warrant further discussion. Some of these genes were found to impact the plant architecture, resulting in increased shoot and flower branching, a more compact growth habit, and a shorter flowering time. Engineering such variations can thus facilitate the development of new tomato varieties that are better suited to indoor farming and have higher crop yields than those at present. Furthermore, we have discussed tomato plants that have been gene edited to increase their tolerance to various pathogens and viruses, including *Oidium neolycopersici*, *Phytophtora infestans*, and TYLCV. Additionally, gene-edited tomatoes are being developed to exhibit greater tolerance to drought or salt/osmotic stress.

The widespread application of CRISPR/Cas methodologies for precise genetic modification will facilitate the development of tomato plants that are more tolerant to the multiple stresses associated with the effects of human-driven climate change. These new varieties will not only be suitable for controlled greenhouse conditions but also for outdoor cultivation in order to maintain or even enhance the yield under adverse environmental conditions. On the other hand, the use of CRISPR/Cas could enable the design and production of new tomato varieties with better-quality fruits enriched in vitamins and other essential or bio-healthy compounds. However, in many countries, the regulatory management of crops generated using NGTs does not differ from that of crops produced using conventional genetic improvement techniques, such as mutagenesis, while in other countries, the regulation of NGT crops parallels that of GMOs. This has resulted in regulatory oversight, making it difficult to generalize scientific advances aimed at improving food security in developing countries.

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7. ANNEX II: Other publications

- **Olha Yaroshko** (2021). Achievements in genetic engineering of *Amaranthus* L. representatives. *International Journal of Secondary metabolite* **8**, 172-185. doi: 10.21448/ijsm.925737.
- **Olha Yaroshko**, Mykola Kuchuk (2021). Transient expression of reporter genes in cultivars of *Amaranthus caudatus* L. *Biotechnol Acta* **14,** 53-63, doi: 10.15407/biotech14.04.053.

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Research Article

Achievements in Genetic Engineering of *Amaranthus* L. Representatives

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Abstract: Despite the fact that in the modern world more than a thousand edible plants are used for food, only 3 staple cereal crops are grown worldwide: wheat, rice, and maize. Growing a limited number of crops often causes many problems: ranging from the loss of biodiversity, due to the constant cultivation of the same monocultures in the same areas, to the deterioration of soil quality. A way out of this situation is the selection of new untraditional and neglected plants that could grow in a wide range of temperatures, produce high yields and at the same time have a balanced amino acid composition. Pseudocereals of the genus *Amaranthus* L. meet these criteria. Amaranth grain and plant raw materials are used in many industries: food, medicine, cosmetics.
Modern technologies do not stand still. Along with traditional methods of plant breeding, the rapid pace of development involves genetic engineering of plants, which allows the process of creating improved plants to be speeded up several times.
The purpose of this study is to analyze and systematize the achievements in the field of regeneration and genetic transformation of representatives of the *Amaranthus* genus. The results can be used for a practical application: the genetic transformation of species of the genus *Amaranthus* and other close genera of plants.

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1. INTRODUCTION

Amaranth is a high-yielding plant. From 1 plant it is possible to obtain more than 5,000 seeds. Moreover, amaranth has a uniquely balanced amino acid composition that ensures easy digestion. Amaranth is a rich source of protein and essential amino acids, deficits of which cannot be compensated by traditional agricultural crops.

Furthermore, amaranths are used in medicine. Amarantin substance ($C_{29}H_{31}N_2O_{19}$) was derived from some species of amaranth (*A. caudatus* L., *A. tricolor* L., *A. cruenthus* L.) (Yaacob *et al*., 2012). Amarantin relates to alkaloids-betalains. It has useful antioxidant properties in the human organism (Burd, 2006).

Due to the fact that amaranths are indifferent to the type of soil and are drought-resistant, they are grown as a grain crop in countries with a temperate climate (Western Europe), as well

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as in hot-climate countries, where many traditional crops grow poorly: Mexico, the USA, African countries, India.

Given that amaranth is one of the main food crops in India and Africa, has a unique rich amino acid composition with a high nutritional value, and can serve as a source of biologically active substances for further use in medicine, amaranth plants have undergone improvements for many decades using hybridization, selection and mutagenesis methods.

In recent years, the chemical composition of plants and some agronomic properties have begun to improve using biotechnological methods, namely genetic engineering. Genetic engineering methods make it possible to improve not only the useful properties of a plant, but also to provide additional useful characteristics during plant transformation.

Since it is known that the percentage of *Agrobacterium* - mediated transformation of plants is often low, usually even before this transformation possible ways of obtaining a large number of transformed plants from a single parent plant are consequently worked out. One of the optimal methods of rapidly increasing the number of plants is considered to be direct regeneration of plants *in vitro* conditions.

Therefore, we first consider the main achievements related to obtaining regenerants of amaranths *in vitro*.

2. ACHIEVEMENTS IN REGENERATION OF *AMARANTHUS L.* SPECIES

To date, there have been many studies on the regeneration and callus formation of amaranth. Basically, the researchers who obtained calluses, had as primary objective their use as a source of secondary metabolites and other valuable substances. In this connection, the largest number of studies devoted to amaranths have had a biochemical orientation.

Amin and colleagues verified the possibility of obtaining the *Amaranthus gangeticus* L. callus. The leaves, stems and roots were used as initial explants. The scientists observed the formation of calluses in 99.7% ± 0.2% of explants which were derived from stem calluses on MS medium supplemented with 2.0 mg/l NAA(α-Naphthalene acetic acid) + 1.0 mg/l BA (6-benzylaminopurine) (Amin *et al*., 2015).

The group headed by Bennici studied the morphogenesis and growth of calluses. As an object of investigation, they chose lines of several species: *A. caudatus* L., (PI490458, AMES15114, AMES5461), *A. cruentus* L. (434, 622, AMES2248, AMES2247, PI511731, PI777913), *A. hybridus* L. (1047), *A. hypochondriacus* L. (1221, 718, 674, 722, 412, PI540446). The stem segments derived from 15-day sprouts were used for explants (Bennici *et al*., 1997).

Callus tissue was obtained from the explants of the lines *A. caudatus* L. (Bennici *et al*., 1997), *A. cruentus* L. (Bennici *et al*., 1997) and *A. hypochondriacus* L. (Bennici *et al*., 1997) on MS medium with the addition of 2.3 μM 2.4-D (2,4-dichlorophenoxyacetic acid) + 2.3 μM KIN (kinetin); NAA from 0.5 μM to 5.4 μM + BA from 0.4 μM to 13.3 μM.

The callus formation was observed in 100% of explants, with the exception of two lines of *A. caudatus* L. and three lines of *A. cruentus* L. and *A. hypochondriacus* L.. Different concentrations of NAA + BA did not induce callus formation on the *A. caudatus* explants line AMES5461, while 5.4 μM NAA + 13.3 μM BA caused callus formation only in 43% of PI490458 *A. caudatus* L. explants. *A. cruentus* L. lines formed calluses in percentage ratios of less than 100%: AMES2247, 71% on MS medium, with addition of 5.4 μM NAA + 4.4 μM BA; PI511731, 60% on MS medium with addition of 2,4-D + KIN and 67% on MS medium with addition of 5.4 μM NAA + 13.3 μM BA; PI477913 – 75% on 2,4-D + KIN and 79% on MS medium with addition of 5.4 μM NAA + 4.4 μM BA.

Plant regenerants were obtained for *A. hybridus* L. (line 1047) and for *A. hypochondriacus* L. (line 674). The rate of regeneration was low – 8.5% (*A. hybridus* L.) and 14.3% (*A. hypochondriacus* L.). Regenerants were also obtained for *A. hybridus* L., *A. hypochondriacus* L., *A. cruentus* L. on MS medium with addition of 2.7 μM NAA + 2.5 μM 2iP (N^6-(2-isopentenyl)adenine), 2.7 μM NAA + 2.3 μM KIN. The regenerants of *A. cruentus* L. line 434 and 1034 were obtained on MS medium with addition of 2.7 μM NAA + 4.4 μM BA. The general conclusion of the authors was as follows: the absolute majority of species and lines of amaranths are able to form calluses on most media tested by the authors (almost 100% of callus formation). There was no clear connection between regeneration of shoots and the use of growth regulators. This is due to the strong influence of the genotype of plants on organogenesis. Amaranths have high levels of cytokinins (auxins), which inhibit regeneration processes. The authors believe that the best stimulator of amaranth regeneration was BA.

Mousumi Biswas and colleagues conducted experiments aimed at obtaining calluses for further isolation of betacianins from them (Biswas *et al*., 2013). The biggest volumes of callus synthesizing betacianins were obtained from explants of stem origin on MS medium supplemented by NAA (0.25 mg/l) + BA (2 mg/l). In addition, researchers found red-purple amaranthine pigment in the callus lines, 2 new yellow pigments and 18 other biologically active phenylpropanoids. A new betaxanthin has been identified and a methyl derivative of arginine betaxanthin was also identified. Pigments were purified by size exclusion chromatography (Biswas *et al*., 2013).

Flores and colleagues studied the formation of callus and regeneration for the *A. hypochondriacus* L., *A. cruentus* L. and *A. tricholor* L. species. They observed a rapid growth of calluses and abnormal roots on *A. hypochondriacus* L. and *A. cruentus* L. leaf disks on MS medium in the presence of 0.1–1.0 mg/l of 2.4-D. At higher levels (1.0–10.0 mg/l) of 2,4-D, embryo-like structures formed from the surfaces and veins of the leaf discs. Shoots were formed from hypocotyl derivative callus on the medium B5 + 0.1 mg/l NAA and 0.1–1.0 mg/l ZEA (zeatin). Lower ratios of ZEA/NAA stimulated the formation of roots from hypocotyl segments (Flores *et al*., 1982).

Gajdošová, with a team of researchers, selected the ideal conditions for the regeneration and cultivation of *Amaranthus cruentus* L. 'Ficha' and *Amaranthus hybridus* (Gajdosova *et al*., 2007; Gajdosova *et al*., 2013) 'K-433'. As explants, they used epicotyls with the first pair of leaves, hypocotyls, roots and segments of the leaves of 10-day seedlings. For both species studied, the most effective media for direct regeneration from epicotyls were MS_{30}, supplemented with 5 mg/l BA + 0.01 mg/l NAA, MS_{30} supplemented with 1 mg/l TDZ (thidiazuron), MS_{30} supplemented with 3mg/l TDZ + 0.01 mg/l NAA. The most effective medium for induction of callus was MS_{30} with 6 mg/l NAA + 0.1 mg/l BA (for *Amaranthus cruentus* L. 'Ficha') and MS_{30} + 2 mg/l 2.4 D + 0.5 mg/l BAP (for *Amaranthus hybridus* L. "K-433"). The authors made the following conclusions: in order to obtain regenerants, it is necessary to use mediums with a high cytokinin content: auxins; amaranths are characterized by a high callus forming ability, almost 100% on all tested mediums; regenerants were obtained only from epicotyl segments; the ability to regenerate strongly depends on the genotype, age of plants and used types of explants; the overall regeneration frequency was low (Gajdosova *et al*., 2007; Gajdosova *et al*., 2013).

Flores and colleagues investigated the regeneration ability and the callus formation of the following species: *A. hypohondriacus* L., *A. cruentus* L., A. *tricolor* L.. Parts of the hypocotyls were used as explants. The regeneration was indirect (first, callus tissue was obtained). The scientists concluded that the optimal medium for regeneration is B5 supplemented with 0.1mg/l NAA + 0.1–1.0 mg/l ZEA. The callus tissue was obtained from leaf discs of *A. hypohondriacus* L. and *A. cruentus* L. Intensive growth of the callus was observed on MS_{30} medium with 0.1–1

mg/l 2,4-D. However, after addition to the MS_{30} medium of 0.2 mg/l BA + 2 mg/l NAA and 10% coconut water, they observed shoot induction from callus tissue (Flores & Teutonico, 1986).

The team of researchers headed by Bennici intended to obtain regenerants for the following species: *A. hypohondriacus* L., *A. cruentus* L., *A. hybridus* L., *A. caudatus* L. As explants, hypocotyls were used. Regeneration was obtained for 2 species as a result: *A. hypochondriacus* L. (MS_{30} + 3 mg/l BA + 1 mg/l NAA), *A. caudatus* L. (MS_{30} + 3 mg/l KIN + 0.3 mg/l IAA (indole-3-acetic acid). The percentage of regeneration was low (26%). At the same time as the main objective of obtaining regenerants, researchers obtained a callus tisssue. Rapid and intensive callus formation from hypocotyl explants was observed for *A. cruentus* L. (6 mg/l NAA + 0.1 mg/l BA) and *A. hybridus* L. (6 mg/l 2.4-D + 0.1 mg/l KIN (Bennici *et al*., 1992). Arya and colleagues chose *A. paniculatus* L. as an object of research. Parts of the inflorescence were used as explants. When transferring the explants on the MS_{30} medium with 8–15 mg/l KIN or MS_{30} + 5–10 mg/l BA, secondary inflorescences were formed from stems and leaves of the primary inflorescence buds (Arya *et al*., 1993). Bui van Le and colleagues received regenerants of *A. edulis* L. from thin cell layers. For experiments, they used thin slices (0.2–0.4 mm) of cotyledons, hypocotyls, roots, tissues from the apical and sub-apical areas. Explants were obtained from 7-day seedlings (Bui van Le *et al*., 1998). Regenerants were obtained solely from tissues taken from the apical and sub-apical zone. Only callus tissue was obtained from all other types of explants.

Initially, embryonic buds were formed from the tissues of the apical and sub-apical zone on a medium of MS_5 + 2 μM TDZ + 10 μM of CPPU (forchlorfenuron). These embryonic buds were then transferred on MS_5 + 10 μM BAP for elongation of stems (Bui van Le *et al*., 1998). Tisserat and Galletta obtained only callus tissue for *A. gangenticus*, *A. hypochondriacus*, *A. caudatus* L., *A. viridis* L., *A. retroflexus* L. (Tisserat & Galletta, 1988). Callus tissue was obtained by Yaacob and colleagues. Callus was obtained for further extraction of biologically active substances using leaves, stems, roots on MS_{30} + 1.5 mg/l IAA + 0.5 mg/l of ZEA or MS_{30} + 1 mg/l IAA medium (Yaacob *et al*., 2012).

A team of researchers headed by Bagga, studied the regeneration ability and callus formation of *A. paniculatus* L. The hypocotyls were used as the explants. Regeneration of 1-2 shoots from one end of the hypocotyls explants was obtained on medium B_5 + 1ppm KIN + 1 ppm NAA; on medium B_5 + 0.5 mg/l KIN + 0.1 mg/l NAA numerous buds formed (10–14 pieces), from which stems developed later. Intensive callus growth was observed on medium B_5 + 1 mg/l GA_3 (gibberellic acid) + 1 mg/l KIN + 1mg/l 2,4-D (Bagga *et al*., 1987).

Jofre-Garfias and co-authors obtained embryos from the cotyledons of *A. hypochondriacus* L. cv. Azteca on medium MS_3 + 10% coconut milk and MS_3 + 10μM 2.4-D (Jofre-Garfias *et al*., 1997). Pal and colleagues obtained *A tricolor* regenerants from hypocotyls and epicotyls of 7-day seedlings on MS_{30} + 13.2 μM BA +1.8 μM NAA (Pal *et al*., 2013 a). In another study, Pal argued that he and his colleagues received regenerants of *A. spinosus* from the culture of "hairy" roots. Regenerants were obtained on MS_{30} medium without growth regulators (spontaneous regeneration) and on MS_{30} medium + 2 mg/l ZEA (Pal *et al*., 2013 a).

Swain and his colleagues obtained *A. tricolor* regenerants from the culture of "hairy" roots. Regenerants were obtained (on MS_{30} medium without growth regulators (spontaneous regeneration) and on MS_{30} medium + 2 mg/l ZEA (Swain *et al*., 2009; Swain *et al*. 2010).

For clarity, the achievements in the field of callus formation and regeneration is presented in tabular form (Table 1).

Table 1. Achievements in amaranth regeneration.

Species of amaranth, cultivar, hybrid, line	Most effective medium for regeneration	Type of explants, age	Authors, year of publication Title
A. cruentus L. 'Ficha', *A. hybridus* 'K-433'.	MS_{30} + 5 mg/l BA + 0.01 mg/l NAA	epicotyls with 1st pair of leaves	(Gajdošová *et al*., 2013)
A. cruentus L. 'Ficha', *A. hybridus* 'K-433'.	MS_{30} + 1 mg/l TDZ, MS_{30} + 3mg/l TDZ + 0.01mg/l NAA	epicotyls of 10-day seedlings	(Gajdošová *et al*., 2007)
A. hypohondriacus L., *A. cruentus* L., *A. tricolor* L.	B_5 + 0.1mg/l + 0.1-1.0 mg/l ZEA	hypocotyls	(Flores *et al*., 1982)
A. hypohondriacus L., *A. cruentus* L.	MS_{30} + 2mg/l NAA + 0.2 mg/l BA + 10% coconut water	hypocotyls (non-direct regeneration), leaf discs	(Flores & Teutonico, 1986)
A. caudatus L., *(PI490458, AMES15114, AMES5461),* *A. cruentus* L., *434, 622, AMES2248, AMES2247, PI511731, PI477913)* *A. hybridus* L. *1047,* *A. hypochondriacus* L.), *1221, 718, 674, 722, 412, PI540446)*	MS_{30} + 2.7µM NAA+ 2.5µM 2iP, 2.7µM NAA + 2.3µM KIN). 2.7 µM NAA + 4.4 µM BA	stems	(Bennici *et al*., 1997)
A. caudatus L., *A. hypochondriacus* L.	MS_{30} + 0.3 mg/l IAA + 3mg/l KIN; MS30 + 1mg/l IAA + 3mg/l BA; MS_{30} + 6mg/l 2,4-D + 0.1 mg/l KIN; MS_{30} + 6mg/l NAA + 0.1 mg/l BA	hypocotyls (non-direct regeneration)	(Bennici *et al*., 1992)
A. paniculatus L.	MS_{30} + 8-15 mg/l KIN or 5-10 mg/l BA; MS_{30} + 0.5 – 10mg/l 2.4-D+ 0.5 – 10 mg/l NAA	inflorescence	(Arya *et al*., 1993)

Table 1. Continued.

A. edulis L.	MS30 + 2 µMTDZ, MS30 + 10µM CPPU	thin cell layers, obtained from the apical and sub-apical meristems of 7-day seedlings	(Bui van Le et al., 1998)
A. paniculatus L.	B5 KIN (0.5 ppm) and NAA 0.1 ppm), B5 + 1 mg/l GA3 + 1 mg/l KIN + 1 mg/l 2,4-D.	hypocotyls	(Bagga *et al*., 1987)
A. hypochondriacus, cv 'Azteca' L.	MS_{30} + 13.2 µM BA+1.08µM NAA	epicotyls and hypocotyls 7 day seedlings	(Jofre-Garfias *et al*., 1997)
A. spinosus L.	MS_{30}, MS_{30} +2mg/l ZEA	"hairy" roots	(Pal *et al*., 2013 b)
A. tricolor L.	MS_{30} + 13.2 µM BA +1.8 µM NAA	epicotyls and hypocotyls 7-day seedlings	(Pal *et al*., 2013 a)
A. tricolor L.	MS_{30}, MS30 +2mg/l ZEA	"hairy" roots	(Swain *et al*., 2009; Swain *et al*. 2010)
A. gangenticus L.	MS_{30} + 2 mg/l NAA + 1 mg/l BA	Leaves, stems, roots	(Amin *et al*., 1993)
A. cruentus L.	MS_{30} + 1.5 mg/l IAA + 0.5 mg/l ZEA; MS_{30} + 1 mg/l IAA	Leaves, stems, roots	(Yaacob *et al*., 2012)

3. ACHIEVEMENTS IN THE TRANSFORMATION OF AMARANTHUS SPECIES AND FUTURE PROSPECTS

The next step after obtaining regenerated plants is genetic transformation. The number of studies devoted to genetic transformation of *Amaranthus* is rather small.

So far, it is reported that genetically transformed parts or whole plants of amaranth have been obtained by two different methods: *Agrobacterium*-mediated transformation and transformation using the "floral-dip" method.

The *Agrobacterium* – mediated transformation method was developed on the basis of a natural process. Wild soil bacterium *Agrobacterium rhizogenes* or *tumefaciens* is able to infect plants, causing the appearance of "hairy" roots (*A. rhizogenes*) or tumors – crown galls (*A. tumefaciens*). At the same time as the infection process, the transfer and integration of two groups of genes into the plant genome occurs. Genetically modified *Agrobacterium* transfers the genes of interest or selective genes needed by humans into the plant's genome.

The first experiments on the transformation of amaranths were unsuccessful (De Cleene & De Ley, 1976). At present, it has been proved that transgenic amaranth plants can be obtained through *Agrobacterium*-mediated transformation. But still there are very few studies devoted to amaranth transformation.

Transgenic roots were obtained for *Amaranthus tricolor* L. (Swain *et al*., 2010) and *A. spinosus* L. (Pal & Swain, 2013). Transgenic plants were obtained for *A. hypochondriacus* L. and *A. tricolor* L. (Pal & Swain, 2013; Swain *et al*., 2009; Swain *et al*., 2010), *A. retroflexus* L. (Taipova & Kuluev, 2015), *A. viridis* L. (Taipova & Kuluev, 2015), *A. cruentus* L. (Taipova & Kuluev, 2015).

There is no information on the transformation of *A. caudatus*, varieties of which are also used in agriculture.

Transgenic roots were obtained for *A. tricolor* L. plants by Swain and colleagues (Swain *et al*., 2010) and for *A. spinosus* L. by Pal and colleagues (Pal & Swain, 2013). The transformation of amaranths was carried out using a wild strain of *Agrobacterium rhizogenesis* A4. Research group Taipova, Kulaev and others obtained transgenic roots for *A. cruenthus* L. from epicotil segments (Taipova *et al*., 2019 a; Taipova *et al*., 2019 b).

Positive results were also obtained in the transformation of amaranth species using strains of *Agrobacterium tumefaciens*. Jofre-Garfias and co-authors transformed the Azteca variety of *A. hypochondriacus* L. They used the vector from *Agrobacterium tumefaciens* with marker genes (Jofre – Garfias *et al*., 1997). Transgenic *A. tricholor* L. was obtained by two different groups of scientists – Swain and colleagues and Pal with co-authors (Swain *et al*., 2009; Pal *et al*., 2013). A team of researchers headed by Pal used a vector with marker genes.

Taipova and Kulaev obtained regenerated transformed plants from epicotil explants after *Agrobacterium*-mediated transformation (Taipova *et al*., 2019 b; Taipova & Kuluev, 2018).

Castellanos-Arévalo with colleagues obtained transgenic *A. hypochondriacus* L. and *A. hybridus* L. from "hairy" roots culture after transformation by *A. rhizogenes* strains ATCC 15834, A4 and HRI. They obtained transgenic plants with *rolB*, *bar*, *gfp*, *uid*A genes (Castellanos-Arévalo *et al*., 2020).

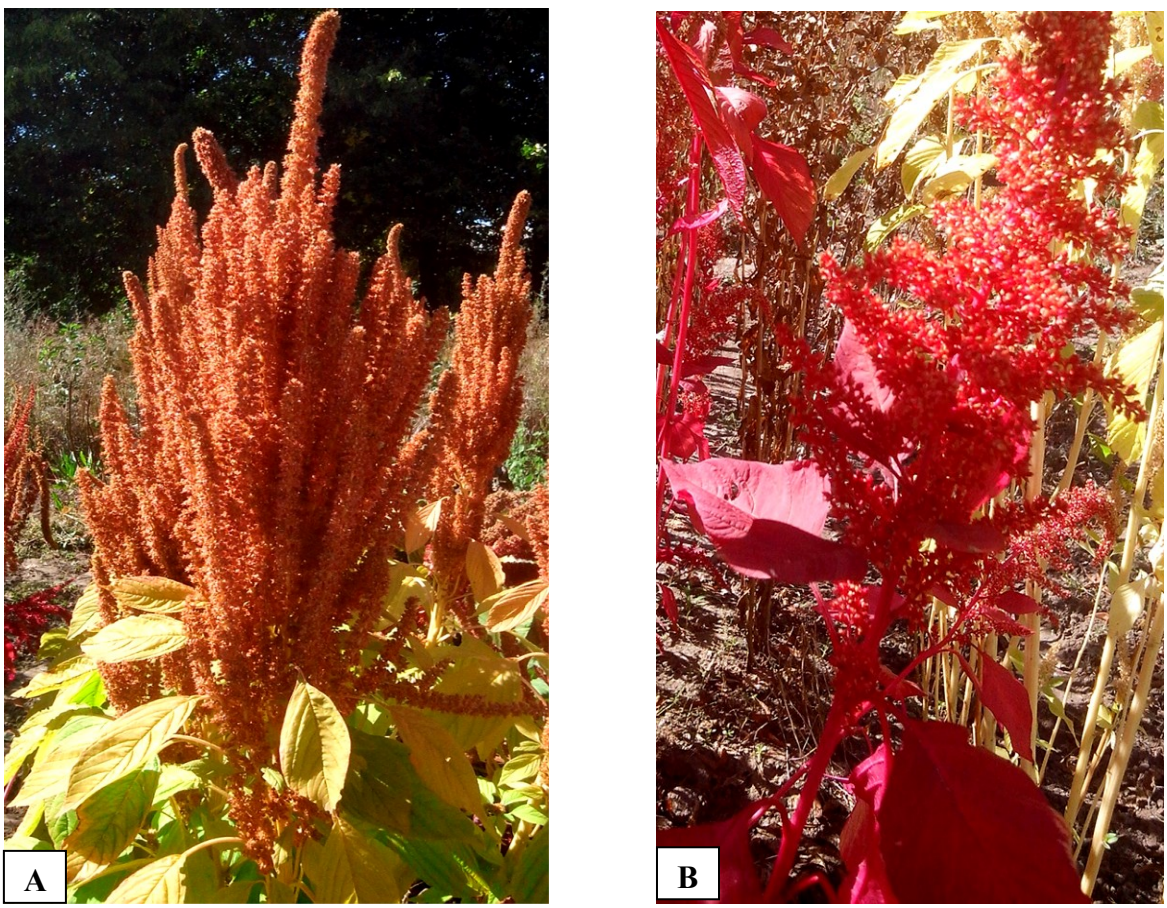
There are also 3 studies devoted to amaranth transformation through inflorescences by the "floral-dip" method – Umaiyal Munusamy and co-authors. They used a vector with selective genes (Munusamy *et al*., 2013).

Another group of researchers – Taipova and Kuluyev – carried out experiments on the transformation of *A. retroflexus* L. (Kuluev *et al*., 2017; Taipova & Kuluev, 2015; Taipova *et al*., 2019 a; Taipova *et al*., 2019 b), *A. viridis* (Kuluev *et al*., 2017; Taipova & Kuluev, 2015;

Taipova *et al*., 2019 a; Taipova *et al*., 2019 b), *A. cruentus* L. (Kuluev *et al*., 2017; Taipova & Kuluev, 2015; Taipova *et al*., 2019 a; Taipova *et al*., 2019 b). They used inflorescences of immature plants for transformation by the "floral-dip" method.

Yaroshko, Kuchuk and co-authors obtained transgenic plants of *A. caudatus* L. local cultivars Karmin and Helios with *bar* gene, after transformation by the "floral-dip" method (Yaroshko *et al*., 2018; Yaroshko & Kuchuk, 2018) (Figure 1).

Figure 1. *Amaranthus caudatus* L. cultivars Helios (A) and Karmin (B).



"Floral-dip" method protocols are described in detail in the articles of several authors (Curtis, 2005; Martins *et al*., 2015). This method was first successfully applied to *Arabidopsis thaliana* transformation (Clough & Bent, 1998; Bent, 2006; Harrison *et al*., 2006; Zhang *et al*., 2006). In addition, successfully transformed by this method were *Brassica rapa via* (Hu *et al*., 2019), *Setaria* (Saha & Blumwald, 2016; Sood & Prasad, 2017; Van Eck, 2018; Van Eck & Swartwood, 2015), rice (Ratanasut *et al*., 2017), *Schrenkiella parvula* (Wang *et al*., 2019), sugarcane (Mayavan *et al*., 2015), tomato (Sharada *et al*., 2017), *Eustoma grandiflorum* (Fang *et al*., 2018). The researchers from the Umaiyal Munusamy group, as well as Taipova and Kuluyev, assured that they had obtained viable transgenic seeds.

Yaroshko and Kuchuk obtained transgenic plants of *A. caudatus* L. and hybrids *A. caudatus* L.x *A. paniculatus* L. after floral-dip transformation (Yaroshko & Kuchuk, 2018). The researchers Murugan and Sathishkumar obtained only transgenic callus for *A. trisis* (Murugan & Sathishkumar, 2016*)*, after transformation of parts of leaves with the *Agrobacterium tumefacies* strain EHA 105 harboring pCAMBIA 1301 (Murugan & Sathishkumar, 2016). The achievements in the field of amaranth transformation are presented in tabular form below (Table 2).

Table 2. Achievements in amaranth transformation.

Species of amaranth, cultivar	Parts of plants used for transformation	Strain of *Agrobacterium* used for transformation	Result	Authors, year of publication
A. hypochondriacus L. *"Azteca"*	germs and cotyledons	*A.tumefaciens* pgv2260 (pEsc4 with genes of *npt* II (neomycin phosphotransferase gene) - kanamycin resistance and *uid*A (gene of *β*-glucuronidase)	transgenic plants	(Jofre – Garfias *et al*., 1997)
A. tricolor L.	internodes and leaf blades	*A. rhizogenes* A4	transgenic plants	(Swain *et al*., 2009)
A. tricolor L.	internodes and leaf blades	*A. rhizogenes* A4, LBA9402	"hairy" roots, transgenic plants***	(Swain *et al*., 2010)
A. spinosus L.	internodes and leaf blades	*A. rhizogenes* LBA9402	"hairy" roots, transgenic plants***	(Pal & Swain, 2013)
A. tricolor L.	epicotyls	*A. tumefaciens EHA 105, LBA 4404 (p35SGUSINT* with genes of *npt II* - kanamycin resistance and *uid*A for each strain)	transgenic plants	(Pal *et al*., 2013)
Amaranthus L.*	inflorescence of adult plants	*A. tumefaciens AGL1 (p5b5, p5d9, p5f7* with gene of *hph* (gene codes hygromycin-B-phosphotransferase protein)	transgenic plants	(Munusamy *et al*., 2013)
A. trisis Willd. (trisis is the synonym of *Amaranthus dubius Mart. ex Thell.*	segments of leaf explants	*A. tumefacies strain EHA 105 harbouring pCAMBIA 1301*	transgenic callus	(Murugan & Sathishkumar, 2016)

Table 2. Continued.

A. retroflexus L.	inflorescence of adult plants	*A. tumefaciens* strain AGL0, which contained gene construction in the vector pCAMBIA 1301 (with ARGOS-like gene from *A. thaliana* (ARL)	transgenic plants	(Kuluev *et al*., 2017)
A. cruentus L.	epicotyls	*A. tumefaciens* strain AGL0, which contained gene construction in the vector pCAMBIA 1301 (with ARGOS-like gene from *A. thaliana* (ARL)	transgenic plants	(Taipova *et al*., 2019)
A. caudatus L.cv. Karmin, cv. Helios	inflorescence of adult plants	*A. tumefaciens* strain GV3101 (with *uid*A and *bar* (phosphinothricin N-acetyltransferase) genes)	transgenic plants	(Yaroshko *et al*., 2018)
A. caudatus L.	hypocotyls	*A. rhizogenes* A4	"hairy" roots	(Yaroshko & Kuchuk, 2018)

Note: * – name of amaranth species not stated;
** – name of *Agrobacterium* strain not stated;
*** –authors did not provide enough results in the publication that would confirm exactly the fact of obtaining transgenic plants.

Thus, at the moment, transgenic amaranth plants have been already obtained with selective genes, marker genes and genes of interest. Research into the transformation of amaranth continues. In the near future, transgenic amaranths may appear that have an improved biochemical composition and new useful properties.

4. CONCLUSION

Amaranth is unique plant. Its nutritional value and optimal amino acid composition have already been evaluated in many countries around the world. In Western Europe, the plant has already gained popularity and it is possible find products with amaranth on the shelves of supermarkets. In Ukraine, we also have a small range of products that include amaranth.

At the moment, plant regenerants have been obtained for 9 species of amaranth (*A.cruentus* L., *A. hybridus* L., *A.hypohondriacus* L., *A. caudatus* L., *A. paniculatus* L., *A. edulis* L., *A. spinosus* L., *A. tricolor* L., *A. gargenticus* L.), transformed plants for 6 species (*A.hypohondriacus* L., *A. tricolor* L., *A. spinosus* L., *A. retroflexus* L., *A. viridis* L., *A. cruentus* L.), transformed organs and tissues for 4 species (*A. spinosus* L., *A. trisis* L., *A. caudatus* L., *A. tricolor* L.).

As can be understood from our previous experimental work and the work of other authors, there are difficulties in achieving regeneration for many species of amaranths. If regenerants are obtained, the percentage of regeneration does not exceed 30 percent, which is clearly not enough for further obtaining transformed plants after agrobacterial transformation.

Therefore, other transformation techniques are being developed, for which it is not necessary to obtain regenerated plants. The alternative transformation method is called “floral-dip’. According to published studies, transformed plants have been obtained using this method.

At present, mainly transgenic amaranth plants have been obtained, which were transformed by agrobacteria that carried vectors containing selective genes. Only one group of authors obtained transgenic plants with not only selective genes, but also genes of interest.

In the near future, a greater number of amaranth species will be obtained, which will present additional useful features, such as, for example, protein synthesis, which can be used in medicine. The authors hope, that in the near future, amaranth will achieve the position of a recognized niche of the food and medicine industries.

Declaration of Conflicting Interests and Ethics

The author declares no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the author.

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EXPERIMENTAL ARTICLES

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TRANSIENT EXPRESSION OF REPORTER GENES IN CULTIVARS OF *Amaranthus caudatus* L.

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Local cultivars of *A. caudatus*: Helios and Karmin were used as plant material. Amaranth is a new pseudocereal introduced in Ukraine. The plant biomass of amaranth is used in medicine, food industry and cosmetology industry.

Aim. The purpose of the work was to identify the optimal conditions for the transient expression of reporter genes in *Amaranthus caudatus* cultivars.

Methods. Biochemical and microscopy methods were used in the following work. Seedlings and adult plants of different age were infiltrated with agrobacterial suspensions separately (genetic vector pCBV19 with a *uidA* gene and genetic vector pNMD2501 with a *gfp* gene in *Agrobacterium tumefaciens* GV3101 strain).

Results. Transient expression of the *uidA* and *gfp* genes was obtained in amaranth plants after conduction series of experiments. The most intensive transient expression of *gfp* and *uidA* genes was observed in seedlings infiltrated at the age of 1 day. The maximum fluorescence of the GFP protein was observed on 5^{th}–6^{th} days.

Conclusions. It was shown that the cultivar Helios was more susceptible to agrobacterial infection than the cultivar Karmin. The effectiveness of *Agrobacterium* mediated transformation was from 16% to 95% for the Helios cultivar and from 12% to 93% for the Karmin cultivar. The obtained results indicate that the studied amaranth cultivars can potentially be used for obtaining transient expression of target genes and synthesizing target proteins in their tissues in the future.

Key words: *Amaranthus*, *uidA*; *gfp*; *Agrobacterium;* transient expression.

The term "transient gene expression" refers to the expression of genes that are expressed shortly after the nucleic acid of bacteria has been introduced into eukaryotic cells. During transient expression, there is no integration of foreign genes into the nuclear genome of plants. In this way the genetic material that has been integrated into plant cells is not inherited by offsprings during the sexual reproduction of plants [2].

Transient gene expression in plant systems has several advantages over stable expression. Transient expression technology does not need the regeneration of transformed tissues or organs, nor does it influence the plant genome stability. This technology allows accelerating the experiments, so the functions of the target genes can be studied 4–10 days after the incorporation of foreign genes in the plant cells. Transient expression allows studying the gene functioning in non-sterile conditions [1, 2]. Transient expression also permits protein interactions to be studied [3, 4].

Transient gene expression can be achieved via several methods of delivering of genetic information. One of which these is agroinfiltration which allows infiltrating many plants at the short time period. Moreover, several genetic vectors (with different genes) can be used for the infiltration of a single plant [5, 6].

Genetic constructs used to obtain transient expression often carry a gene where the target gene is transcriptionally fused to a reporter gene (for example, the *green fluorescent* gene (*gfp*)).

Reporter genes are those genes that encode proteins, the presence of which can be quickly detected by the appearance of fluorescence or specific staining of transformed tissues when stained with a dye. In turn, reporter proteins encoded by reporter genes can help to detect the localization of target proteins in certain organs, tissues, or organelles of plant cells [2].

Mainly, *gfp* and *uidA* are used as reporter genes. The presence of the *gfp* gene is detected by the appearance of green fluorescence of transformed plant tissues under blue rays. The presence of the *uidA* gene is detected by staining plant tissues in blue color when they come into contact with a specific dye. Genetic vectors with these genes are often used in *Agrobacterium*-mediated transformation, when it is necessary to obtain a transient or stable gene expression [2].

The choice of a particular reporter gene for use in experiments should be based on data from the localization in the plant cell of the product encoded by the reporter gene. Thus, the GFP protein encoded by the *gfp* gene is an effective reporter protein in experiments where the localization of the target protein is in the nucleus [7, 8], cytoplasm [9, 10], plasma membrane [10], Golgi apparatus [11], endoplasmic reticulum [9, 11], tonoplasts [12], mitochondria [13] and chloroplasts [11], while reporter yellow fluorescent protein (YFP) and mCherry are used to assess the localization of target protein in peroxisomes [6, 14].

Representatives of the *Amaranthus* genus were the objects of our investigation. The choice is due to the wide use of amaranth plant raw materials in various industries: food industry; pharmaceuticals, agriculture. Improving the quality of amaranth using genetic engineering methods offers considerable potential.

Representatives of *Amaranthus* genus have unique amino acid composition and are rich in biologically active compounds (squalene and amarantin). Squalene has anticancer and wound healing properties. Amarantin has an antioxidant effect [16]. The properties of *Amaranthus* can be improved using biotechnological methods to produce biologically valuable substances (for example, squalene and amarantin).

The possibility of transient expression of the *gus* gene was shown in our previous work for adult *A. caudatus* plants [16, 48]. Yet, there has been no information about obtaining the transient expression of the *gfp* gene in representatives of the *Amaranthus* genus. We show here for the first time the results of the transient expression of the *gfp* gene for the *Amaranthus* genus.

Materials and Methods

The objects of the research were cultivars of *Amaranthus caudatus*: Helios and Karmin. The seeds were obtained from the M. M. Grishko Botanical Garden of the National Academy of Sciences of Ukraine.

Plants of different age: 1 day-old seedlings, 10 day-old seedlings, 2 month-old adult plants were used in the experiments. To obtain 1-day-old seedlings, seeds were soaked for one day in water under non-sterile conditions (22–26 °C, 14-hour light period, illumination — 3 000–4 500 lx). To obtain 10-day-old seedlings and 2-month-old plants, seeds were sown in the pots with soil and grown in a greenhouse under the conditions of 22–26 °C, 14-hour light period and illumination — 3 000–4 500 lx.

The aim of the experiments was as follows: to check and evaluate the functioning of the pCBV19 and pNMD2501 genetic vectors of *A. tumefaciens* in *A. caudatus* plant tissues after *Agrobacterium*-mediated transformation; to determine the optimal age of plants for infiltration and to identify the plant's organs and tissues in which the transient gene expression occurs the most intensively.

The vacuum infiltration method [15] and methods for detection of *uidA* [17] and *gfp* genes presence were used to obtain transient gene expression.

Plants of different ages (previously mentioned) were infiltrated with agrobacterial suspensions. The strains GV3101 of *A. tumefaciens* harboring pCBV19 [16] and pNMD2501 genetic vectors separately were used in the work (supplementary material Fig. 1). The genetic vector pNMD2501 was kindly donated by NOMAD Bioscience GmbH (Germany). Genetic vector pCBV19 carried *uidA* gene, genetic vector pNMD2501 carried *gfp* gene.

The steps of preparation of agrobacterial suspension were described in the author's previous article [16].

Plants were infiltrated in a flask with a medium containing the agrobacterial suspension for 5 min, at 22–24 °C in a vacuum chamber under pressure of 0.1 mPa.

Detection of the *uidA* gene (β-glucuronidase activity) was carried out by histochemical assay on the 5th day after infiltration in the presence of substrate, X-gluc (5-bromo-4-chloro-3-indolyl-β–D-glucuronide) [17].

The leaves of the infiltrated plants and control plants (negative control) which were

not infiltrated, were taken and incubated in a histochemical buffer (50 mM sodium phosphate, pH 7.0; 50 mM EDTA, pH 8.0; 0.5 mM $K_3Fe(CN)6$; 0.5 mM $K_4Fe(CN)6$; 0.1% Triton X-100; 1 mM X-gluc). The histochemical reaction was stopped after 24h of incubation at 37 °C in the dark, followed by five rinses in 70% ethanol. Leaves of stably transformed *Nicotiana tabacum* plants were used as positive control.

Next, the leaves of adult plants and whole seedlings were placed on microscope slides for observation (Zeiss axiophot fluorescent microscope®, Germany; microscope magnification ×100 and ×200). Beta-glucuronidase protein (GUS) activity was detected visually by the appearance of blue staining of plant tissues. Leaves of stably transformed *Nicotiana tabacum* were used as positive control.

The presence of the GFP protein was detected after 4 days in the seedlings (that were immersed in a suspension of *A. tumefaciens* with genetic vector pNMD2501) and was evaluated visually under light with a wavelength in the range of 365–400 nm (Black ray®, model B 100 AP the ultraviolet lamp.) and a microscope with an attachment with a special filter (Plan-Neofluar). The result was considered as positive by the appearance of green tissue fluorescence. The results were documented by photographing on digital media.

Data collection and statistical analysis

One hundred plants (young seedlings) and 30 plants (2-month-old adult) of each variety were used for each part of the experiment. Namely 100 seedlings of cv. Helios and 100 seedlings of cv. Karmin (1-day-old); 100 seedlings 10-day-old of each cultivar and 30 plants of each cultivar (2-month-old) were infiltrated with suspension of *A. tumefaciens* (harboring pCBV19 genetic vector).

For the experiment of *gfp* expression were used 100 seedlings of cv. Helios and 100 seedlings of cv. Karmin (1-day-old); 100 seedlings 10-day-old of each cultivar; 30 plants of each cultivar (2-month-old) which were infiltrated with suspension of *A. tumefaciens* (harboring pNMD2501 genetic vector). The same quantity of seedlings and adult plants of each variety as mentioned above (for the experiment of transient expression of *uidA* and *gfp* gene) were used as negative control (non-infiltrated with agrobacterial suspension).

The percentage of *uidA*-positive plants for each age group (as a percentage expressed the number of plants in which were detected the presence of *uidA*/*gfp* genes from the total quantity of plants, which were infiltrated) was calculated after obtaining the results. The standard error (SE) and the arithmetical mean (M) were calculated using the Excel program 2007 and the *t*-Student criterion was calculated in the program Statistica in order to determine the accuracy of the obtained results.

Results and Discussion

Transient expression of uidA gene

The histochemical reaction was performed after conducting a series of experiments with infiltration [17]. Large areas of plant tissues stained in blue color were identified. Such staining occurred in plant tissues where the GUS protein was bound with the specific X-gluc substrate. This may indicate that after infiltration, bacterial genes were incorporated into plant cells, DNA was correctly transcribed and a functional GUS reporter protein was synthesized in plant tissues.

The intensity of blue staining varied among the plant groups of different ages, as well as varied the surface areas that were colored in the plants of different ages. In young seedlings (in most of the seedlings which were infiltrated at the age of one day) all parts of the plant (root, hypocotyl and cotyledons) were stained (supplementary material Fig. 2). The percentage of positive gus-stained plants for the cultivar Helios was 95%, for the cultivar Karmin — 93%. The areas in which the reporter protein GUS was synthesized (in 10-day-old seedlings) were mainly along the midrib and occupied most of the surface area of the leaf blade (more than 80%) (Fig. 1, supplementary material Fig. 3). The percentage of gus-positive plants (which were infiltrated at the age 10 days) for the cultivar Helios was 61.26%, for the cultivar Karmin — 41.55%.

In plants that were infiltrated at the age of 2 months, small areas stained in blue color were revealed only in the region of the midrib. The percentage of gus-positive plants was for the cultivar Helios — 16% and for the cv. Karmin 12% (supplementary material Fig. 4). These results indicate that very young seedlings 1-day-old of both cultivars (Helios and Karmin) were the most susceptible to agrobacterial infection. In seedlings that were infiltrated at the age of 10 days and 2 months, the cv. Helios displayed a higher susceptibility to agrobacterial infection. Perhaps this is due to the peculiarities of the biochemical composition

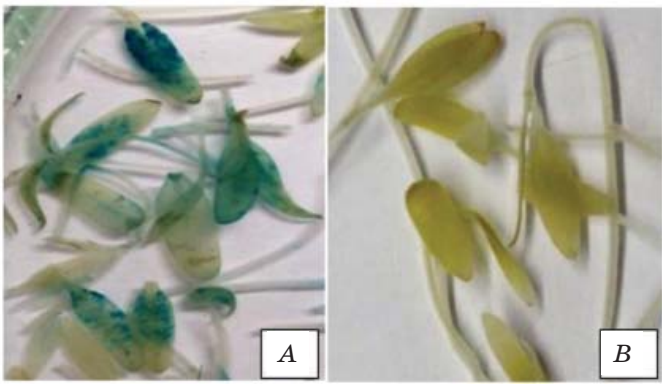


Fig. 1. **15-day-old seedlings of *A. caudatus* cv. Helios after the histochemical reaction:**
A — seedlings infiltrated with *A. tumefaciens* harboring genetic vector pCBV19;
B — non-infiltrated control seedlings

Supplementary material Fig. 1. **Schematic representation of the T-DNA site of the pNMD2501 genetic vector:**
LB — left border sequence; RB — right border sequence; Nos pro — nopaline synthase promoter; Nos ter — nopaline synthase terminator; 35S prom — promoter of cauliflower mosaic virus gene (CaMV); Ocs — octopine synthase terminator; Ω — regulatory sequence enhancer; gfp — green fluorescent protein gene; P19 — gene of protein P19 (suppressor of gene silencing)

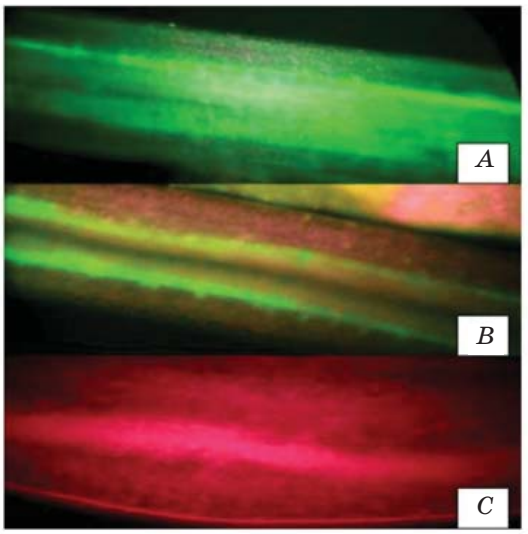
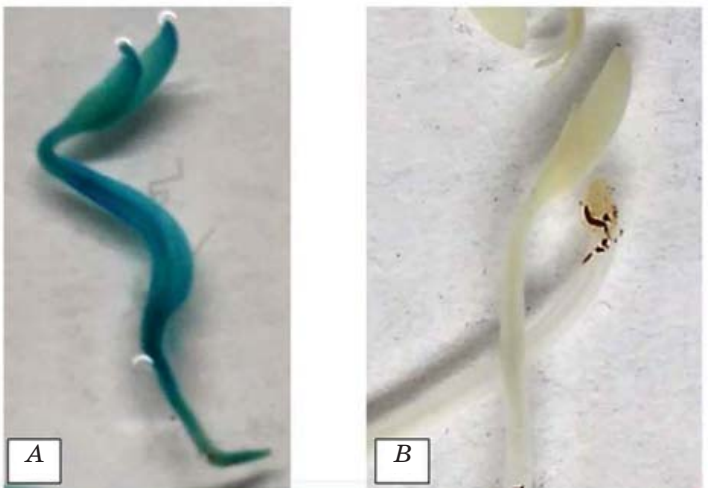


Fig. 2. **Hypocotyls of *A. caudatus* seedlings (15-day-old) which were infiltrated with *A. tumefaciens* harboring genetic vector pNMD2501 under UV light (*A*, *B*)** (magnifection ×200)
A — cv. Helios; *B* — cv. Karmin); *C* — hypocotyls of non-infiltrated control plant (cv. Helios) (magnifection ×200)



Supplementary material Fig. 2. **Seedlings of *A. caudatus* cv. Helios (6-day-old) after the histochemical reaction:**
A — seedlings infiltrated with *A. tumefaciens*, genetic vector pCBV19; *B* — non-infiltrated control seedlings of cv. Helios

of plants. The cultivar Karmin has a higher content of betacyanins than the cultivar Helios. Betacyanins can reduce the transformation efficiency of *Agrobacterium* [16].

Transient expression of gfp gene

The next stage of the work was the analysis of plants that were infiltrated *via A. tumefaciens* harboring genetic vector pNMD2501, carrying the *gfp* gene. The results of transient expression of the *gfp* gene were analyzed visually using an ultraviolet light and were considered as gfp-positive when green fluorescence of tissues appeared (Fig. 2–5).

In seedlings of both cultivars (which were infiltrated at the age of 10 days), green fluorescence was observed in hypocotyls and at the edges of leaf blades (Fig. 2, 3).

Microscopic examination revealed that the most intense transient expression of the *gfp* gene occurred in the vascular bundles of the hypocotyl and in the midrib of the leaf blade

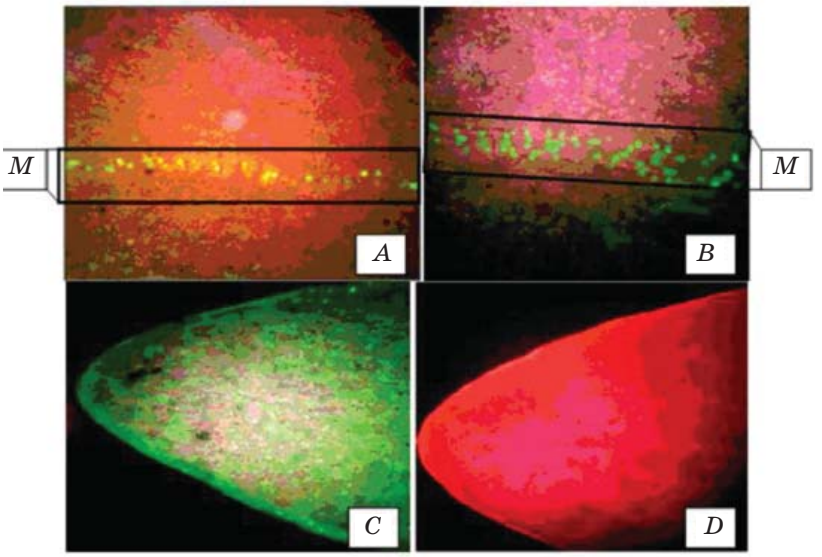


Fig. 3. **Cotyledonous leaves of *A. caudatus* seedlings (15-day old) which were infiltrated with *A. tumefaciens*, genetic vector pNMD2501 under UV light (*A*, *B*, *C*):**
A — cv. Helios (magnifection ×100); *B* — cv. Karmin (magnifection ×100); *C* — top of the cotyledonous leaf cv. Helios (magnifection ×100); *D* — leaf of non-infiltrated control plant (cv. Helios); *M* — area of midrib

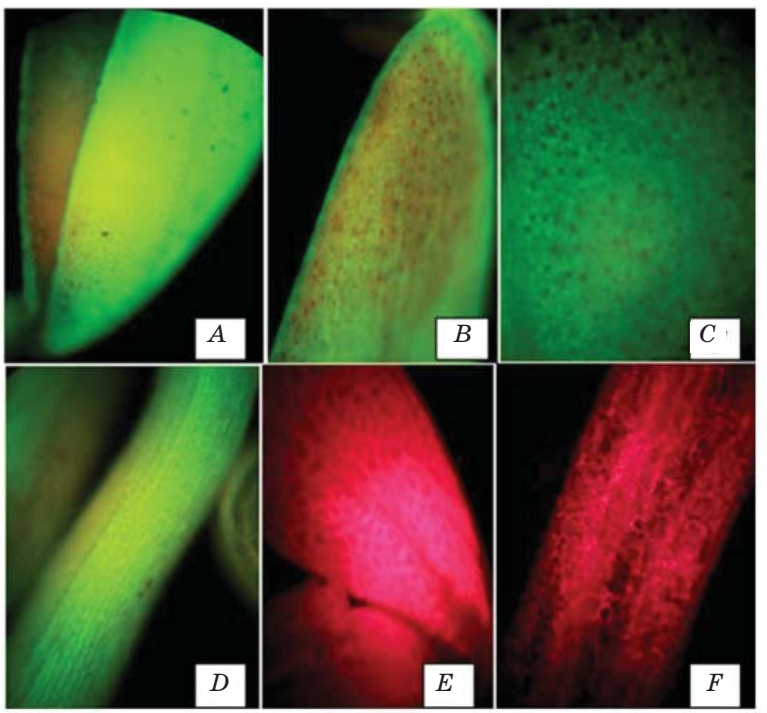
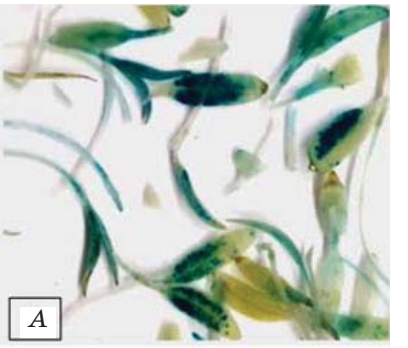
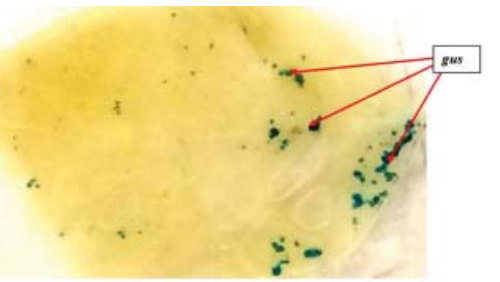


Fig. 4. **Seedlings of cv. Helios which were infiltrated with *A. tumefaciens* harboring genetic vector pNMD2501 (6-day old) under UV light (*A*, *B*, *C*, *D*):**
A — petiole and lower part of cotyledonous leaves (magnifection ×100); *B* — top of the cotyledonous leaf (magnifection ×100); *C* — hypocotyl (magnifection ×200); *D* — hypocotyl (magnifection ×100); *E* — cotyledonous leaves of non-infiltrated control plant (magnifection ×100); *F* — hypocotyl of non-infiltrated control plant (magnifection ×200)



Supplementary material Fig. 3. **Seedlings of *A. caudatus* cv. Karmin (15-day-old) after the histochemical reaction:** *A* — seedlings infiltrated with *A. tumefaciens*, genetic vector pCBV19; *B* — non-infiltrated control seedlings)



Supplementary material Fig. 4. **Leaf of *A. caudatus* variety Karmin (2-month-old) after the histochemical reaction (plant was infiltrated with *A. tumefaciens*, genetic vector pCBV19), gus —areas, where activity of β-glucuronidase was detected**

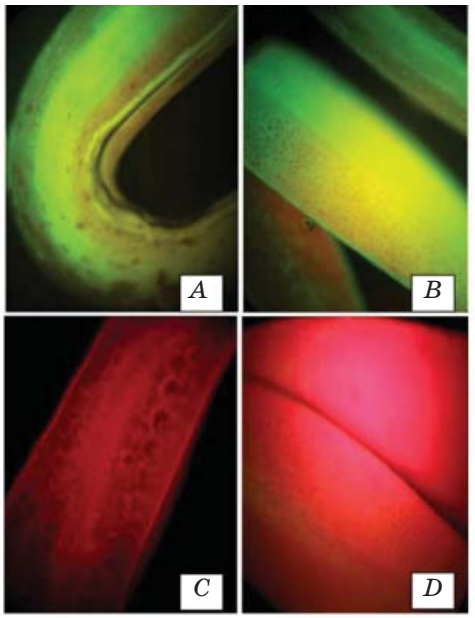


Fig. 5. **Seedlings of cv. Karmin which were infiltrated with *A. tumefaciens* harboring genetic vector pNMD2501 (6-day old) under UV light (*A*, *B*):**
A — hypocotyls (magnifection ×100);
B — hypocotyl and part of cotyledonous leaves (magnifection ×100); *C* — hypocotyl of non-infiltrated control plant (magnifection ×200); *D* — part of non-infiltrated control cotyledonous leaf (magnifection ×100)

(Fig. 3).

In seedlings, which were infiltrated at the age of 1 day (both cultivars), intensive green fluorescence was detected in all organs (root, hypocotyl, cotyledonous leaves) (Fig. 4, 5).

Microscopy of the seedlings which were infiltrated at the age of one day, revealed a very intense green glow in all tissues of the aforementioned seedling organs (Fig. 4, 5).

It should be noted that in plants that were infiltrated at the age of 2 months, only a points of green glow were visible on the leaf blades in the region of the central vein. So, we obtained transient expression of the *gus* and the *gfp* genes in all plants of all experimental groups.

Agrobacterial infiltration of the youngest seedlings (1 day-old) turned out to be more effective. Expression was more abundant in young plant tissues which intensively synthesized proteins. In plants that infiltrated at an older age, expression occurred mainly in vascular bundles and leaf midrib (seedlings infiltrated at the age of 10 days), or only in vascular bundles and leaf midrib (plants that infiltrated at the age of 2 months). It was found that amaranth cultivars have different susceptibility to agrobacterial infection. The cultivar Helios was more susceptible to agrobacterial infiltration (Fig. 6).

The number of plants in which were confirmed the expression of the *gus* gene was significantly or highly significantly different from those group of plants which were not infiltrated with *Agrobacterium*.

So far, transient gene expression has been obtained in the following plants: *Arabidopsis thaliana* [18, 19], *Capsicum annuum* [20, 21]; *Catharanthus roseus* [22, 23]; *Cucumis sativus* [24]; *Fragaria* × ananassa [12], *Fragaria vesca* [25], *Glycine max* [26], *Helianthus annuus* [27], *Juglans regia* [28, 29], *Lactuca sativa* [30], *Fagopyrum esculentum* [31], *Brasica napus* [32].

There is currently a great deal of experimental work on obtaining transient gene expression in *Nicotiana benthamiana* and review articles that mention the successful transient expression of various genes in *Nicotiana benthamiana* [33, 34].

According to the latest literature, the reporter *gfp* gene has been used in *Agrobacterium*-mediated transformation of the following plant species: *Fagopyrum esculentum* [31], *Setaria italic* [35], *Nicotiana tabacum* cv. Bright Yellow 2 [36], *Vigna unguiculata* [37], *A. hypohondriacus* and *A. hybridus* [38], *Oryza sativa* cv. Kitaake [39], *Setaria italica* [40], *Nicotiana benthamiana* [41], *Solanum lycopersicum* [41], *Solanum tuberosum* [41], *Physalis peruviana* [41].

The *uidA* reporter gene was used in *Agrobacterium*-mediated transformation of the

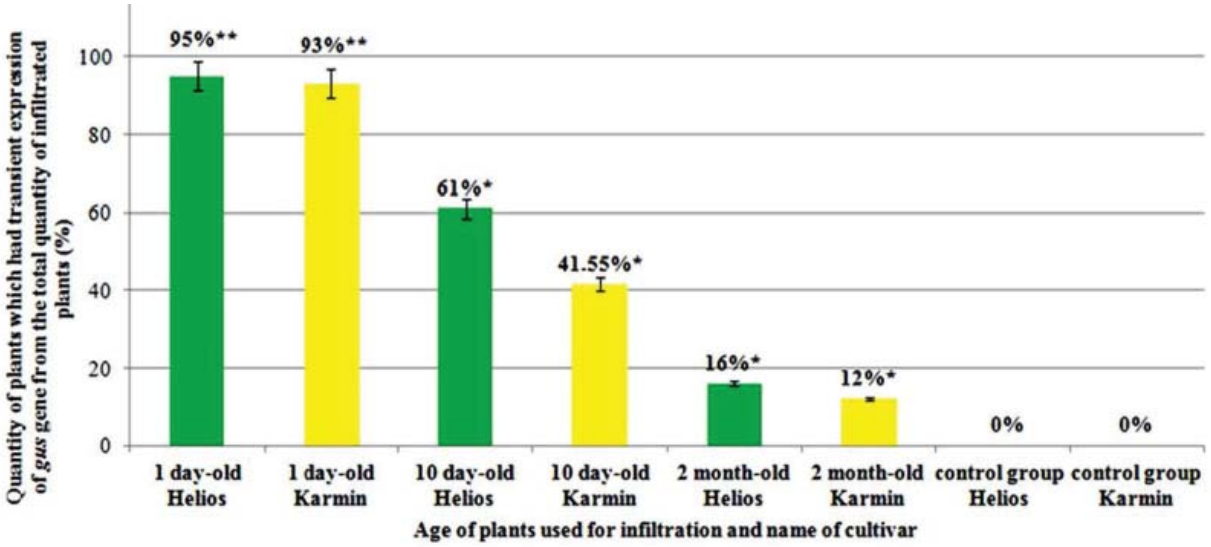


Fig. 6. **Effectiveness of vacuum infiltration of different age plants with agrobacterial suspension (*A. tumefaciens* harboring genetic vector pCBV19), expressed as a percentage:**
values showing significantly differences between the study groups and control groups are marked with asterisks * (* significant ($P < 0,05$); ** — highly significant ($P < 0,01$))

following plant species: *A. hypohondriacus* and *A. hybridus* [38], *Oryza sativa* cv. Kitaake [39], *Setaria italica* [40], *Cannabis sativa* [42].

There is only one report of transient gene expression in representatives of *A. hypochondriacus* and *A. hybridus* [38], indicating insufficient investigation in this sphere.

In our experiments, the most intensive fluorescence of the GFP protein was observed in seedlings infiltrated at the age of one day in all parts of plant. GFP fluorescence was observed also in the hypocotyls (areas of vascular bundles) and in cotyledon leaves (mainly point fluorescence in the area of midrib). In the leaves of 2-month-old plants fluorescence of GFP protein was observed with maximum fluorescence observed on 5^{th}–6^{th} days.

After infiltration of whole amaranth plants under vacuum with a suspension of *Agrobacterium tumefaciens* harboring the genetic vector pCBV19 and histochemical reaction, positive results of β-glucuronidase activity were obtained for two cultivars (Karmin and Helios) (blue areas). Gus-positive areas were located mainly in the middle and lateral veins. This may indicate that the most sensitive tissues to agrobacterial transformation and in which active protein synthesis occurs are the central and lateral veins [43, 44] (supplementary material Fig. 4).

It is known that when interpreting the results of the histochemical reaction, a number of problems may arise. For example, residues of live *Agrobacterium* suspension left on the surface of untransformed plant tissues can lead to false-positive results in standard histochemical analysis and thus may complicate the analysis of transformation results [16]. Usage of genetic vectors with intron increases the reliability of the histochemical analysis. An intron was presented in the pCBV19 genetic vector, to enable the histochemical reaction to take place only in plant tissues and this ruling out the possibility of a false positive result in the presence of agrobacterial contamination.

Chimeric genetic constructs have been used successfully in the *Agrobacterium*-mediated genetic transformation of several plants: *Spinacia oleracea* [45], *Momordica dioica* [46], *Spinacia oleracea* [47].

Our results of transient expression of the *uidA* gene after infiltration were not positive for all cultivars of *Amaranthus caudatus*. This may be due to differences in biochemical composition of the various cultivars, which in turn may affect susceptibility to *Agrobacterium* infection. In the leaves, β-glucuronidase activity was detected in the central vein. Our results of localization of the *gus* gene in plant tissues and organs during transient expression are similar to those obtained by Jun Jasic [44].

Conclusions

The optimal conditions for the transient expression of reporter genes in *Amaranthus caudatus* cultivars were determined. The most intensive transient expression of *gfp* and *gus*

genes was observed in seedlings which were infiltrated with agrobacterial suspensions at the age of one day. Maximum fluorescence of GFP protein was observed on 5^{th}–6^{th} days. It was shown that cultivar Helios was more susceptible to agrobacterial infection than the cultivar Karmin. The effectiveness of agrobacterial transformation was from 16% to 95% for the Helios cultivar and from 12% to 93% for the cultivar Karmin.

The obtained results indicate that the studied amaranth cultivars can potentially be used in the future for obtaining transient expression of target genes in their tissues and synthesis of target proteins in their tissues.

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