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Agrobacterium-Mediated Transformation of Tomato Using a Cotyledonary Explant System

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ABSTRACT

Tomato was among the first crops to be transformed through *Agrobacterium tumefaciens*, and the use of cotyledon explants, cut from young sterile seedlings and induced to form shoots on selective medium, remains one of the commonest routes to a transgenic plant. This narrative review describes how that system works and what the published literature says about its weak points. It covers the biology of T-DNA transfer, the choice of strain and binary vector, the preparation and wounding of explants, co-cultivation, selection and regeneration, and the molecular checks used to confirm that a regenerated plant is transgenic. Reported efficiency depends on genotype, explant age and position, and on how a laboratory handles the interval between infection and selection, so figures from different papers are rarely comparable. Regeneration from cotyledons can also yield plants with altered ploidy, and some regenerants escape selection. The review sets out what a well-reported protocol would state, and notes that evidence on several variables rests on single laboratories. It reports no new experimental data and does not validate any protocol.

Keywords: *Agrobacterium tumefaciens*; Tomato; Cotyledon explant; Genetic transformation; Regeneration; Binary vector; Narrative review

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Article Class: Narrative Review

1. Introduction

Tomato is a convenient crop to engineer. It is diploid, it has a well-characterised genome (1), it regenerates readily from cultured tissue, and it has long served as a model for fleshy fruit biology (2, 3). Soon after the first reports that *Agrobacterium tumefaciens* could carry foreign genes into plant cells (4, 5), tomato became one of the first crop species to be transformed in this

way (6, 7). Its early place in the history of the technology, including the antisense work on fruit softening (8, 9), means that the cotyledon protocol is now taught almost as a rite of passage in plant biotechnology laboratories.

Familiarity breeds a certain carelessness. Methods sections describing cotyledon transformation are often short, and they tend to cite an earlier paper rather than



state the conditions that were actually used. Readers who then try to reproduce the work find that the details they need, such as seedling age, preculture, bacterial density and the timing of selection, are exactly the ones that were left out. Reported efficiencies vary widely, and it is not always clear whether the variation reflects biology or bookkeeping (10, 11).

This article is a narrative review of the cotyledon explant system as it appears in the published literature. We ask what is established about each stage of the procedure, where the evidence comes from a single laboratory or a single cultivar, and what a methods section should say so that another group could repeat the work. No local or experimental data are reported, and the article does not endorse any particular protocol over another. The aim is to describe the method as the literature records it, not as any one laboratory remembers it.

2. Literature Review

2.1 How *Agrobacterium* Transfers DNA

Agrobacterium tumefaciens causes crown gall by moving a segment of its tumour-inducing plasmid, the T-DNA, into plant cells, where it becomes part of the plant genome (12). The process is initiated when the bacterium senses signals released by wounded plant tissue. Phenolic compounds such as acetosyringone switch on the virulence genes (13), and the proteins they encode excise a single-stranded copy of the T-DNA and escort it into the host cell (14). Once inside, the T-DNA depends on plant proteins to reach the nucleus and to integrate (15, 16).

For biotechnology the important fact is that everything between the T-DNA borders can be replaced. The oncogenes can be removed and a gene of interest and a selectable marker put in their place,

which is how the disarmed strains and vectors in use today came about (4, 5). The plant cell does most of the work of integration, and the outcome, usually a small number of insertions that are sometimes truncated or rearranged, is therefore partly out of the experimenter's hands (17, 18). The plant is also not a passive recipient: infection triggers defence and stress responses that can work against transformation, and the old picture of a bacterium simply injecting DNA has given way to one of a negotiation between two organisms (19).

2.2 Strains and Binary Vectors

A major practical advance was the binary vector strategy, in which the virulence functions sit on one plasmid in the bacterium and the T-DNA on a smaller, easily manipulated plasmid (20, 21). Binary vectors made cloning far simpler and are now the default, with a large and well-organised literature on their features (22). Helper plasmids and disarmed strains differ in how strongly they induce transfer, and reports suggest that choice of strain matters most for crops and cultivars that are difficult to transform (23, 24).

Promoters and markers complete the construct. The cauliflower mosaic virus 35S promoter, defined by deletion analysis (25), is still widely used to drive both marker and gene of interest, and the beta-glucuronidase reporter gives a rapid visual check that transformation has occurred (26). A kanamycin-resistance marker was used in the first tomato work and has remained common (6, 7).

2.3 Explants and Regeneration

The practical advantage of cotyledons is that they are available in large numbers from sterile seedlings, are physiologically uniform, and regenerate shoots at a high rate in many cultivars. Comparative work in the early 1990s examined the conditions



that govern regeneration and transformation from tomato explants, including the age of the tissue and the composition of the medium (11, 27). Table 1 gathers the stages and the decisions they involve. Subsequent studies pointed to further factors, among them preculture and the use of feeder cells (10), and to protocols described as genotype-independent (28) or especially efficient in a particular cultivar (29, 30).

Regeneration is not purely a matter of dosage. The balance of cytokinin and auxin in the medium steers the tissue between callus and shoot formation, and the basal salts and vitamins shape the response (31, 32). Variation between cultivars is considerable, and a single recessive allele that improves regeneration in the model cultivar Micro-Tom shows how much of the difference may be genetic (33, 34).

2.4 Infection, Co-cultivation and Selection

After excision, explants are exposed to the bacterial suspension and then placed on medium for a period of co-cultivation, during which T-DNA transfer takes place. The conditions in this window, including temperature, light and the presence of an inducer, are among the least consistently reported. Bacteria must then be suppressed so that they do not overgrow the tissue, and a combination of antibiotics has been found to improve the outcome compared with a single agent (35). The same antibiotics can slow regeneration in the plant tissue, so the choice is a compromise. Laboratories rarely say how they reached theirs, and a regime that suits one cultivar may suppress the shoots of another. Wounded cotyledons also tend to darken during co-cultivation as phenolic compounds oxidise, and some protocols add antioxidants for that reason, though the evidence that this helps is uneven and

mostly anecdotal.

Table 1. Stages of a cotyledon-based transformation protocol and the decisions each involves.

Stage	Main decision	What to report
Explant source	Genotype, seedling age, cotyledon position (11, 27)	Cultivar and conditions
Preculture	Medium, hormones, time before infection (10)	Medium and duration
Infection	Strain, vector, inducer, bacterial density (13, 23)	Strain and vector map
Co-cultivation	Length, light, temperature, antioxidants	Conditions in full
Selection	Antibiotic, timing, bacterial control (35)	Agent and onset
Regeneration	Cytokinin and auxin balance (29, 33)	Hormones and passages

Note: Qualitative summary of choices discussed in the cited literature; no stage has a single correct setting.

Selection comes next. Only cells that have taken up and expressed the marker survive on the selective medium, but not all survivors are transformed. Tissue protected by neighbouring resistant cells can persist for a while, and some shoots escape selection altogether. Escapes of this kind are tedious to detect afterwards and cheap to prevent in the first place. The sequence in which selection is applied relative to co-cultivation therefore affects how many false positives reach the rooting stage.

2.5 Beyond Tomato

Success with tomato helped to establish the idea that *Agrobacterium* was not limited to dicotyledonous model plants. Efficient transformation of rice and maize, species once thought out of reach, followed after changes to explants and



inducers (36, 37), and the host range now extends well beyond plants (38). At the other extreme, the floral dip in *Arabidopsis* dispenses with tissue culture altogether (39). These contrasts are a useful reminder that the cotyledon system is a choice, with its own costs, and not a necessity.

3. Materials and Methods

3.1 Scope and Question

The review addresses one question in two parts: what is known about the stages of *Agrobacterium*-mediated transformation of tomato using cotyledon explants, and how reliably do published reports describe those stages? The question was divided into the steps a laboratory has to carry out, from seedling to confirmed transformant. We did not consider transformation of other organs, hairy root systems or transient assays, except where they clarify a point about the cotyledon system.

3.2 Literature Assembly

The review draws on peer-reviewed articles and reviews on the biology of T-DNA transfer, vector design, tomato regeneration and transformation, and methods for characterising transgenic plants. Sources were chosen for relevance, influence in the field and clarity of their methods, and only works published up to 2014 are cited. Literature on other species is used for what it teaches about the mechanism, not as direct evidence for tomato.

We did not run a formal systematic search and we do not claim one. This is a narrative synthesis, and the choice of sources reflects the authors' judgement. The practical consequence is that the review is a better guide to what a good report should contain than to the exact efficiency of any particular protocol. For that reason we avoid quoting efficiency figures from individual papers. Efficiency figures quoted without these details are

hard to compare from one laboratory to the next.

3.3 Appraisal Framework

To judge how far a reported protocol can be trusted and reused, we used four questions. Is the genotype named and the seedling stage described? Are the strain, vector and bacterial density stated, so that the infection can be reproduced (22, 23)? Are selection and antibiotic regimes given with their timing (35)? Are transgenic status and copy number confirmed by methods that match the claim, and is the ploidy of regenerants known (40, 41)? The answers are summarised qualitatively in the next section; no scoring was attempted. A reader choosing a protocol needs to know which parts are mechanism and which are habit.

4. Results

4.1 The Cotyledon Protocol as Described in the Literature

Across the literature the procedure follows a recognisable sequence (Figure 1). Seeds are surface-sterilised and germinated on defined medium. Cotyledons are cut, often with the tips and bases removed so that wounded edges are exposed, and placed on a preculture medium that usually contains a cytokinin. The explants are infected, co-cultivated, and moved to regeneration medium containing a selective agent and an antibiotic against the bacterium. Shoots that form are excised, rooted and then screened (6, 29, 30).

Each step is a place where plants are lost. Some cultivars regenerate poorly, bacteria can overgrow the explants, selection can leave escapes and chimeric shoots, and regeneration from cut tissue can produce plants with altered ploidy (41). The sketch in Figure 1 is meant to show where these losses occur and not how large they are; no published figure is reproduced in it.

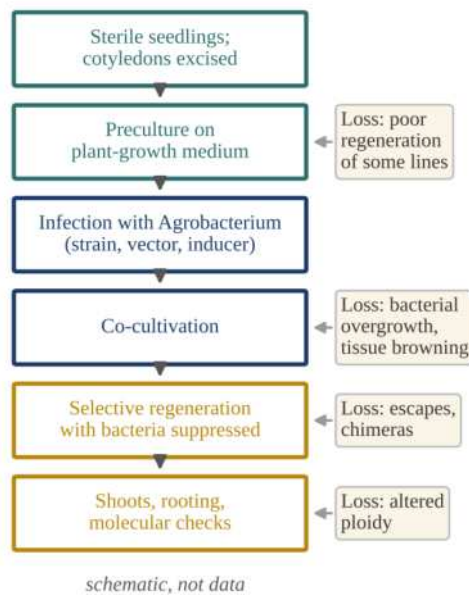


Figure 1. Outline of a cotyledon-based transformation protocol and points of loss (schematic, not data).

4.2 Variables That Shape the Outcome

Table 2 lists the variables that the literature most often links to success or failure. Genotype is the clearest. Some lines regenerate readily and others hardly at all, and attempts to find conditions that work across lines have met with partial success only (28, 33). The age of the seedling and the position of the explant on the cotyledon also matter, as the comparative studies on regeneration showed (10, 27). This is one reason the same cultivar can behave very differently in two laboratories.

The bacterial side is better understood mechanistically than the plant side. The inducer, the virulence background of the strain and the density of the culture all influence how much T-DNA reaches the plant cell (13, 23). Their effect on final transformation frequency is smaller and harder to separate from plant factors, because transfer is rarely the limiting step in a responsive cultivar. In practice, laboratories tend to settle on a combination that works for their material and to change it little.

Table 2. Variables reported to influence transformation outcome in tomato.

Variable	Reported influence	Evidence
Genotype	Large; some lines regenerate poorly (28, 33)	Consistent
Explant age	Younger tissue usually favoured (27)	Moderate
Bacterial strain	Strains differ in virulence (22, 23)	Moderate
Phenolic inducer	Activates vir genes (13)	Strong
Feeder layer	Reported to help in some protocols (10, 11)	Mixed
Antibiotic choice	Affects regeneration as well as bacteria (35)	Moderate

Note: The last column states how strongly the cited literature supports the influence; it is the authors' reading and is not a pooled estimate.

Figure 2 summarises these relationships as a qualitative matrix. It reflects the weight of reporting in the literature and should not be read as a measurement. In particular, an empty cell means that the review found little discussion, not that the variable has been shown to be irrelevant.

	Infection	Co-cult.	Selection	Regen.
Genotype	some			strong
Explant age	some			moderate
Strain / vector	strong	some		
Inducer	strong	moderate		
Antibiotics	some	some	moderate	moderate
Hormones			some	strong

shading shows strength of reported influence; schematic, not data

Figure 2. Qualitative matrix of how strongly variables are reported to affect each stage (schematic, not data).

4.3 Confirming and Characterising Transformants

A regenerated shoot that survives selection is not proof of a transgenic plant. The



usual sequence of checks begins with a reporter assay (26) and PCR on DNA prepared by a standard extraction method (42). These are quick, but they cannot rule out bacterial contamination of the tissue, and they say little about how many copies were inserted. Southern blotting remains the reference for insertion pattern (43), while real-time PCR offers a faster estimate of copy number when properly calibrated (40). Table 3 sets out what each method can and cannot show. The point is not that any one of them is poor, only that they are often asked to answer questions they were never designed for.

Inheritance matters as much as detection. Transgenes can be silenced, and multiple or rearranged copies make that more likely (17, 44). A single T0 plant with a bright reporter signal may therefore give progeny in which expression has faded or segregated unpredictably. Studies that follow expression through at least one generation are consequently much more informative than those that stop at the primary regenerant. Where a study does report segregation of the marker in progeny, it also gives the reader something concrete about the number of independent insertions. None of the individual steps is difficult, but each one offers a way to lose the experiment quietly.

4.4 Reporting Quality

Our reading of the literature, summarised in Figure 3, is that the history of the field is one of technical progress that has outrun the reporting of its details. The core tools were assembled in the 1980s (4, 20, 45), tomato-specific protocols appeared shortly afterwards (6), and by the end of the century a model cultivar and, later, a complete genome sequence were available (1, 34). The methods papers of the 1980s, by contrast, were short and relied on shared laboratory practice.

Table 3. Methods used to confirm and characterise transgenic regenerants.

Method	What it shows	Main limitation
Reporter assay	Expression of a marker (26)	Says nothing about copy number
PCR	Presence of T-DNA sequence	Cannot exclude bacterial carry-over
Real-time PCR	Estimated copy number (40)	Needs careful calibration
Southern blot	Insertion pattern (42, 43)	Slow, needs more DNA
Progeny test	Inheritance and stability (44)	Takes a generation
Flow cytometry	Ploidy of regenerants (41)	Needs fresh tissue

Note: Each method answers a different question; none of them alone establishes a single, stable, expressed insertion.

Several recurring gaps stand out. Genotype and seedling age are often named but the culture conditions are not. The bacterial strain is given, but not the optical density or the duration of infection. Selection is described by agent but not by the interval before it is applied. Ploidy is rarely mentioned in reports of cotyledon-derived plants despite evidence that it depends on genotype and on the procedure (41). Taken one at a time these omissions are small, but they add up to protocols that cannot be repeated without trial and error. A negative result from one cultivar therefore says little about another.

The literature on engineered traits shows what depends on all this. Fruit softening and carotenoid content were both modified through transformation, in the first case by antisense suppression of a cell-wall enzyme (8, 9) and in the second by silencing a regulator of light signalling in the fruit (46). Both achievements rested on a protocol that worked reliably in the cultivar used, and both are a reminder that the method is a means to an end (2, 47) and that its value shows up in what the plants go on to do.

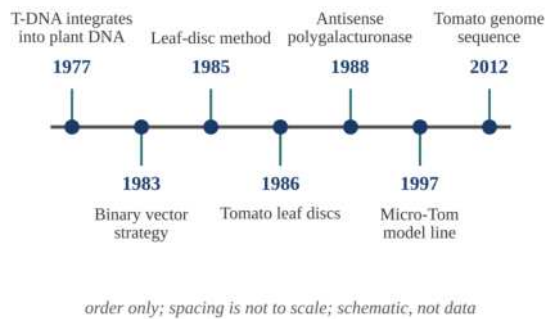


Figure 3. Selected milestones in tomato transformation, in order of publication (schematic timeline, not to scale).

5. Discussion

5.1 Interpretation

The central lesson is that the cotyledon system is robust in principle and fragile in detail. The biology of T-DNA transfer is well understood (18, 24), but outcomes are governed in large part by plant-side factors, particularly genotype and the conditions of regeneration, that are not fully predictable from one laboratory to another (28, 33). A report of high efficiency in one cultivar is therefore best treated as a hypothesis about another.

A second point concerns what efficiency means. Some authors count regenerated shoots, some count shoots that root on selective medium, and some count plants confirmed by molecular tests. These denominators and numerators are not interchangeable, and a sensible comparison needs both to be stated (10, 30). Without that, even careful experiments may not be comparable. Reviews of this kind are most useful when they say plainly where the evidence stops.

5.2 Limitations

This is a narrative review and shares the weaknesses of that form. The selection of sources reflects judgement, no formal search or quality scoring was carried out,

and we avoid giving numbers from individual papers because we could not check them against the primary reports for this article. A reader who needs an efficiency figure should consult the original studies and apply the framework above to them.

The review also leans on work in other species and on mechanistic studies, which are imperfect proxies for the behaviour of tomato cotyledons in culture (36, 39). Much of the evidence on individual variables comes from single laboratories working with a handful of cultivars. We have used it to frame questions, and our statements should be read with that in mind. These points apply with equal force to crops other than tomato.

5.3 Future Work

Three needs stand out. The first is a reporting checklist for cotyledon transformation, short enough that authors would use it, covering genotype, seedling age, strain and density, infection and co-cultivation conditions, selection timing, and the denominators behind any efficiency figure. The second is multi-laboratory comparison of a small number of protocols on a shared set of cultivars, which would separate bacterial from plant effects far better than the present patchwork (16, 24).

The third is routine reporting of ploidy and of inheritance, which would reveal how often regenerants differ from the starting material in ways that matter (41, 44). None of these needs new technology. They need only agreement on what to measure and on what to write down. It is a habit worth keeping even when the outcome is expected.

6. Conclusion

Agrobacterium-mediated transformation of tomato using cotyledon explants is a mature, productive technique whose basic



logic has been clear for decades (12, 14, 18). What the literature does not provide is a dependable picture of how much each step contributes to success, partly because the details of published protocols are incomplete and partly because plant genotype dominates outcomes. That is a modest conclusion, and it is the one the published record will bear. A cautious reading is that the system will work in a responsive cultivar with sensible conditions, and that the efficiency reported elsewhere is a poor guide to what a new line will do. Nothing here argues against the method; it argues for describing it fully.

For the practitioner, the message is to treat the published protocol as a starting point, to record every condition, and to confirm transformants with methods matched to the claim. For the field, the opportunity is modest but real: a shared reporting standard and a few well-designed comparisons would do more for reproducibility than any further protocol variant. The same caution applies to any transformation system that depends on regeneration from cut tissue.

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9. Conflict of Interest

The authors declare no conflict of interest.

10. Author Contributions

T.P. conceived the review and wrote the sections on transformation biology and vectors. J.d.G. wrote the sections on explants, regeneration and selection. W.J. wrote the sections on molecular

confirmation and reporting. All authors revised the manuscript and approved the final version.

11. Ethics Statement

This is a review of published literature. No human participants or animals were involved and no ethical approval was required.

12. Data Availability

No new data were generated. All sources are cited in the reference list.

13. References

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