



Low hanging fruit: harvesting the potential of DNA-free genome editing in red raspberry (*Rubus idaeus*)

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Abstract

Red raspberry (*Rubus idaeus*) is a high-value crop species coveted for its flavour and health benefits. Annual global production is increasing year-on-year and currently exceeds 900,000 metric tonnes. Despite this popularity, raspberry breeding remains laborious and time-consuming, limiting trait improvement and new cultivar development. Genome editing offers a solution to increase the speed and accuracy of plant breeding while reducing costs. The use of DNA-free genome editing, which utilises ribonucleoprotein complexes without transgenic introgression, enables trait enhancement without genetic modification (GM). This is desirable due to enduring negative public sentiment toward GM foods and legislative change in regions such as England. However, there is a large research gap applying DNA-free genome editing methods to raspberry. Non-transgenic genetic improvement in raspberry would be a breeding paradigm shift, enabling trait improvements including increased firmness, improved flavour, disease resistance and optimised plant architecture. This review focuses on the process of DNA-free genome editing, from protoplast source to plant recovery, incorporating new research demonstrating the feasibility of raspberry protoplast isolation and DNA-free editing. We explore remaining technical challenges including the elucidation of protoplast regeneration and the optimisation of editing efficiency. We advocate for better standardisation, integration and clarity of regeneration methods to effectively harvest the opportunities of DNA-free genome editing in crop species.

Key message

DNA-free genome editing offers a path to better breeding without genetic modification. Recent research increases feasibility in raspberry but development of protoplast regeneration methods are needed before commercialisation.

Keywords CRISPR-Cas9 · Genome editing · *Rubus idaeus* · DNA-free · RNPs · Protoplast regeneration

Abbreviations

AI	Artificial Intelligence	DefH9	palindromic repeats
ANS	<i>ANTHOCYANIN SYNTHASE</i>	EU	European Union
BAP	6-benzylaminopurine	EC	Embryogenic callus
BP	Basepair	FC	Friable callus
Cas	CRISPR associated enzyme	GE	Genome editing
CNNs	Convolutional neural networks	GM	Genetic modification
CRISPR	Clustered regularly interspaced short	gRNA	Guide RNA
		IaaM	Indole-3-acetamide monooxygenase
		IBA	Indole-3-butyric acid
		KM8p	Kao and Michayluk formulation 8p
		Mbps	Megabase pairs
		ML	Machine Learning
		MS	Murashige and Skoog
		MT	Metric tonnes
		NAA	Naphthalene acetic acid

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NGS	Next generation sequencing
PDS	<i>PHYTOENE DESATURASE</i>
RNNs	Recurrent neural networks
RNP	Ribonucleoprotein
PEG	Polyethylene glycol
PGRs	Plant growth regulators
SAMase	<i>S-ADENOSYLMETHIONINE</i>
SSNs	Site specific nucleases
STS	<i>STILBENE SYNTHASE</i>
TDZ	Thidiazuron
24D	2, 4-dichlorophenoxyacetic acid
4CL	<i>4-COUMAROYL-COA-LIGASE</i>

Introduction

Basic biology and production

Red raspberry (*Rubus idaeus*) is the most agronomically important species of the *Rubus* genus, a large and diverse group of deciduous shrubs that grow on six continents with 750 described species (Graham and Jennings 2009, 2020; Funt and Hall 2013; Foster et al. 2019). Raspberries grow on

woody stems called canes and have the highest respiration rate among temperate fruit (Funt and Hall 2013). Between 2006 and 2020, production of *Rubus* species rose 63% from 597,504 metric tonnes (mt) to 940,979 mt (FAO 2025). The largest producers were Russia (182,000 mt), Mexico (146,350 mt) and Poland (121,700 mt) (FAO 2025). Such rapid growth has been enabled by year-round berry production, introduction of new cultivars and sustained increases in raspberry consumption (Foster et al. 2019). These factors have also led to an increase in yield in recent years, however to a lesser extent than production (Fig. 1). This suggests there is scope for raspberry production to be further optimised through new breeding technologies to continue improving yields.

Raspberries are often described as a ‘superfood’ (Hancock et al. 2007) and the fruit carry a marketplace premium. Raspberries in the UK cost £16 kg⁻¹ in May 2025, quadruple the value of strawberries (£3.96 kg⁻¹) and are one of the most valuable crops per kg produced in the UK (Department for Environment 2025). Berries including *R. idaeus* contain forty times as many antioxidants as cereal crops, and high levels of vitamins C, A and E. Raspberries are particularly high in anthocyanins which promote the



Fig. 1 Global raspberry production and yield data between 1961 and 2023 (FAO 2025)

neutralisation of reactive oxygen species (ROS). Excessive ROS buildup is associated with many diseases such as cancer, stroke, Alzheimer's, and autoimmune disorders (Hancock et al. 2007). Consequently, in 2014 the European Union (EU) launched the 'QUALIREDFRUITs' project, which aimed to sustainably intensify European production of *R. idaeus*. The project elucidated high antioxidant varieties, plant defence elicitors and microsatellite markers for genetic analysis (European Commission 2013).

Conventional raspberry breeding

Red raspberry varieties available today primarily derive from a few heirloom cultivars developed in the early 20th century, such as 'Latham' and 'Taylor' (Funt and Hall 2013). Common breeding targets include enhanced fruit quality, improved yield, resistance to pests and disease, open fruiting habit, continuous fruiting season (primocane vs. floricanes), machine harvestability, loss of prickles, and adaptation to local conditions. Compared to closely related species such as strawberry, the production and harvest of raspberries remains challenging due to poorly optimised plant architecture (Graham and Jennings 2009), variable berry size and consistency, and very short shelf life (Funt and Hall 2013). Conventional cross breeding has struggled to improve many of these traits due to their polygenic nature, with linkage drag and a lack of consistent genetic markers complicating matters further (Graham and Jennings 2009; Graham et al. 2009; Scolari et al. 2021; Davik et al. 2025).

Breeders primarily seek large berries with excellent sugar-acid balance, glossy deep red colour, uniformity and firmness (Funt and Hall 2013). Within the raspberry cultivars grown today, there remains substantial genetic variation to be exploited through breeding. The relatively small and diploid >300 Mbps (Davik et al. 2022a) genome of raspberry presents a key advantage when applying molecular techniques to breeding: compared to fruit like the octoploid strawberry *Fragaria x ananassa* (Hirakawa et al. 2013), allelic segregation patterns are more predictable in diploids which enables simpler QTL mapping and faster expression of recessive alleles in the breeding program. A small diploid genome also promotes cost efficient genetic mapping and more straightforward genome sequencing. Genome editing is also easier in a diploid genome as only two copies of each target loci need to be edited, and there is less risk of chimerism, off-target effects and masking of recessive edited alleles than in polyploids. Therefore, there is a greater chance of recovering the intended mutated phenotype.

The genome of raspberry was only sequenced in recent years (Wight et al. 2019; Davik et al. 2022b; Price et al. 2023) but provides an excellent resource for genome editing and marker-assisted selection – continued work on this front

will be required for the construction of a potential raspberry pangenome, which would improve breeding accuracy and genomic mapping (Hirsch et al. 2014; Li et al. 2014; Yao et al. 2015).

Traditional raspberry breeding occurs through multiple iterations of hybridisation, conventionally with each cultivar in the cross possessing separate desirable characteristics. Traits of interest often have a highly complex polygenic basis: for example, shelf life is a combination of inter-drupelet coherence, firmness, toughness of drupelet walls and fruit shape. This requires substantial screening due to complex inheritance patterns over multiple generations and this process is also susceptible to linkage drag (Stegmeir et al. 2010). As floricanes take two years to fruit, screening of new cultivars is consequently highly arduous and time consuming. As with many other soft fruits, breeding has historically prioritised fruit quality and yield over pest and disease resistance. Many commercial cultivars are susceptible to *Phytophthora* root rot, raspberry bushy dwarf virus and/or aphid infestation (Funt and Hall 2013). Numerous wild varieties are resistant to such threats and breeding programmes have focused on crossing commercial cultivars with resistant wild relatives.

As a highly heterozygous outbreeding species, red raspberry has further breeding challenges as sexual reproduction results in recombination and segregation at heterozygous loci, introducing genetic variation in offspring. This disrupts favourable allele combinations and results in variable fruit quality and consistency in the next generation (Graham and Jennings 2009). Consequently, commercial raspberry germplasm is vegetatively propagated to maintain the genotype and phenotype of elite cultivars. Ownership of patented germplasm forms the basis of most commercial raspberry breeding operations and is thus carefully guarded. DNA-free genome editing offers an alternative breeding approach where elite cultivars can be directly edited, without interference from segregation and heterozygosity.

Genetic modification and genome editing in raspberry

Genetic modification (GM) has been used since the 1980s to generate new and improved traits in many crop species through the introgression of non-native (transgenic) DNA into plant genomes. It has been successful in increasing global yields and production of key crops like maize and soybean yet remains controversial from some regulatory and consumer standpoints (Wunderlich and Gatto 2015; Turnbull et al. 2021). As a vegetatively propagated heterozygous species, raspberry-specific genetic modification protocols are highly desirable to improve elite phenotypes while maintaining the genetic background. *R. idaeus* is amenable

to genetic modification as a wild host of *Agrobacterium tumefaciens* (Kuzmanović et al. 2015) and was first transformed with β -glucuronidase with successful plant regeneration (Graham and McNicol 1990). An early study with a functional target transformed three cultivars with the S-adenosylmethionine hydrolase (SAMase) gene, which codes for a hydrolytic enzyme that degrades the eventual precursor of ethylene, S-adenosylmethionine (SAM) (Mathews et al. 1995). Reducing SAM expression reduces ethylene levels and hypothetically would increase shelf life, as had been shown in tomato (Good et al. 1994). However, the study did not venture further than validating the introgression of the transgene and establishing plants in soil. Other successful *Agrobacterium* transformations in *R. idaeus* include the transgenic *DefH9-iaaM* gene, which confers parthenocarpy (Mezzetti et al. 2004a), increasing yields 100% (Mezzetti et al. 2004b). *DefH9-iaaM* is a gene fusion product of the regulatory region *DefH9* from snapdragon and the *IaaM* coding region from *Pseudomonas syringae* pv. *Savanstanoi*. More recent work has focused on overexpressing resveratrol synthesis which increased resistance against *Botrytis cinerea* (Zhang et al. 2022a). Resveratrol is a polyphenolic secondary metabolite with antimicrobial properties (Pandey et al. 2014) and expression can be enhanced by introgressing a gene fusion product of 4-coumaroyl-CoA ligase (4CL) and stilbene synthase (STS) (Shin et al. 2011) via agroinfiltration. *Agrobacterium*-mediated transformation is thus feasible in red raspberry but due to their high heterozygosity, transgenes cannot be eliminated from the genome through backcrossing without compromising berry quality. Additionally, public uncertainty and backlash against GM crops (Arntzen et al. 2003; Lang and Hallman 2005; Wunderlich and Gatto 2015; Turnbull et al. 2021) resulted in low consumer confidence and stringent regulations in some regions (particularly the EU) that made commercialisation of GM crops expensive. As raspberry production has a relatively small market capitalisation compared to successful GM crops, it is likely these factors were influential in the failure to get GM raspberries on the market.

CRISPR systems were discovered and initially developed between 1987 and 2012 (Ishino et al. 1987; Jansen et al. 2002; Jinek et al. 2012). Type II CRISPR systems were co-opted to enable programmable DNA mutagenesis in 2012 which has transformed medical and agricultural biotechnology (Sternberg and Doudna 2015; Wang and Doudna 2023). Guide RNA (gRNA) can be designed to target any genomic sequence through sequence complementarity and forms a ribonucleoprotein (RNP) complex with a Cas endonuclease (most commonly Cas9). RNPs trigger a double-strand break at the target loci which is repaired through the error-prone process of non-homologous end joining (NHEJ) or homology-directed repair (HDR) to impair gene function. Cas9

triggers a simple DSB, but various other endonucleases have been developed such as Cas9 nickase (Schiml et al. 2016), which only binds to a single DNA strand for controlled mutagenesis (Pan et al. 2021), or Cas12a, which results in larger deletions (Zetsche et al. 2015, 2017; Hsu et al. 2019; Zhang et al. 2022b). Thus, a flexible toolbox has emerged that permits a wide range of strategies for gene mutagenesis and modifying gene expression. This holds great potential for raspberry breeding, however until recently there had been no peer-reviewed literature on genome editing in this species.

DNA-free genome editing

Overview

DNA-free genome editing, or precision breeding, refers to the production of genetically improved plants where the product does not contain any transgenic genomic DNA. Inbreeding, annual, seed propagated crops can be transformed through classic methods like *Agrobacterium* or particle bombardment where Cas9 protein and gRNA are introgressed into the genome. After gene editing has occurred, transgenes can be removed through backcrossing and genetic segregation in offspring – giving the plant non-transgenic status. As raspberry is highly heterozygous, backcrossing would affect the elite genetic composition. Therefore, the most feasible remaining approach for targeted mutagenesis is protoplast regeneration with RNP transfection. Other transgene-free strategies are arising, including viral and nanomaterial transfection of RNPs (Oh et al. 2021; Ahmar et al. 2021), biolistic bombardment with RNPs (Liang et al. 2019) and shoot apical meristem bombardment (Luo et al. 2025). These techniques are not yet widely used, though significant steps have been made with viral transfection through increased cargo capacity (Qiao et al. 2025) and meristem entry (Li et al. 2021a). These techniques may offer alternative non-transgenic routes to raspberry genome editing in future if development continues outside of model species.

Protoplasts are single plant cells without cell walls, liberated through enzymatic digestion (enzymolysis) of plant tissue such as leaves or callus. Removal of the cell wall facilitates permeability to CRISPR reagents like nucleic acids and proteins. As plant cells are totipotent, a single gene edited protoplast can potentially be regenerated back into a plant. Protoplast regeneration overcomes key issues associated with *Agrobacterium*-mediated transformation – such as the requirement for antibody-mediated selection (which requires introgression of an antibiotic resistance gene) and multiple cloning steps to generate binary vectors

(Marx 2016). Critically, this technique enables non-transgenic genome editing without integration of foreign DNA into the plant genome (Fig. 2). However, protoplast-based methods have several major disadvantages; transfection and regeneration efficiency are highly species-dependent and consequently there is no unified method for whole-plant regeneration (Yue et al. 2021; Reed and Bargmann 2021). This requires extensive custom method development for each species of interest and adaptation for different varieties/cultivars of the same species (Phan et al. 1997; Jeong et al. 2021).

Nonetheless, the non-transgenic potential of modern genome editing techniques has reassured some previously stringent regulators to support genetic improvement in food crops. The Precision Breeding Act (2023) has been a regulatory paradigm shift in England, permitting the development and commercialisation of plants with advantageous

DNA changes that could theoretically have been produced by natural mutagenesis. The EU has reached a provisional agreement to follow a similar approach (European Council 2025) which would transform the regulatory environment for precision bred crops across the European continent. This is welcome news for European raspberry breeders, as gene edited cultivars could be bred in the EU and globally marketed in permissive regions including North America, China, India and most of Latin America (Fernández Ríos et al. 2025).

Protoplast isolation

Protoplast were first isolated enzymatically in 1960 using fungal cellulases (Cocking 1960; Davey et al. 2005). This basic method has endured, and most protoplast extraction protocols use high osmoticum solutions to plasmolyse

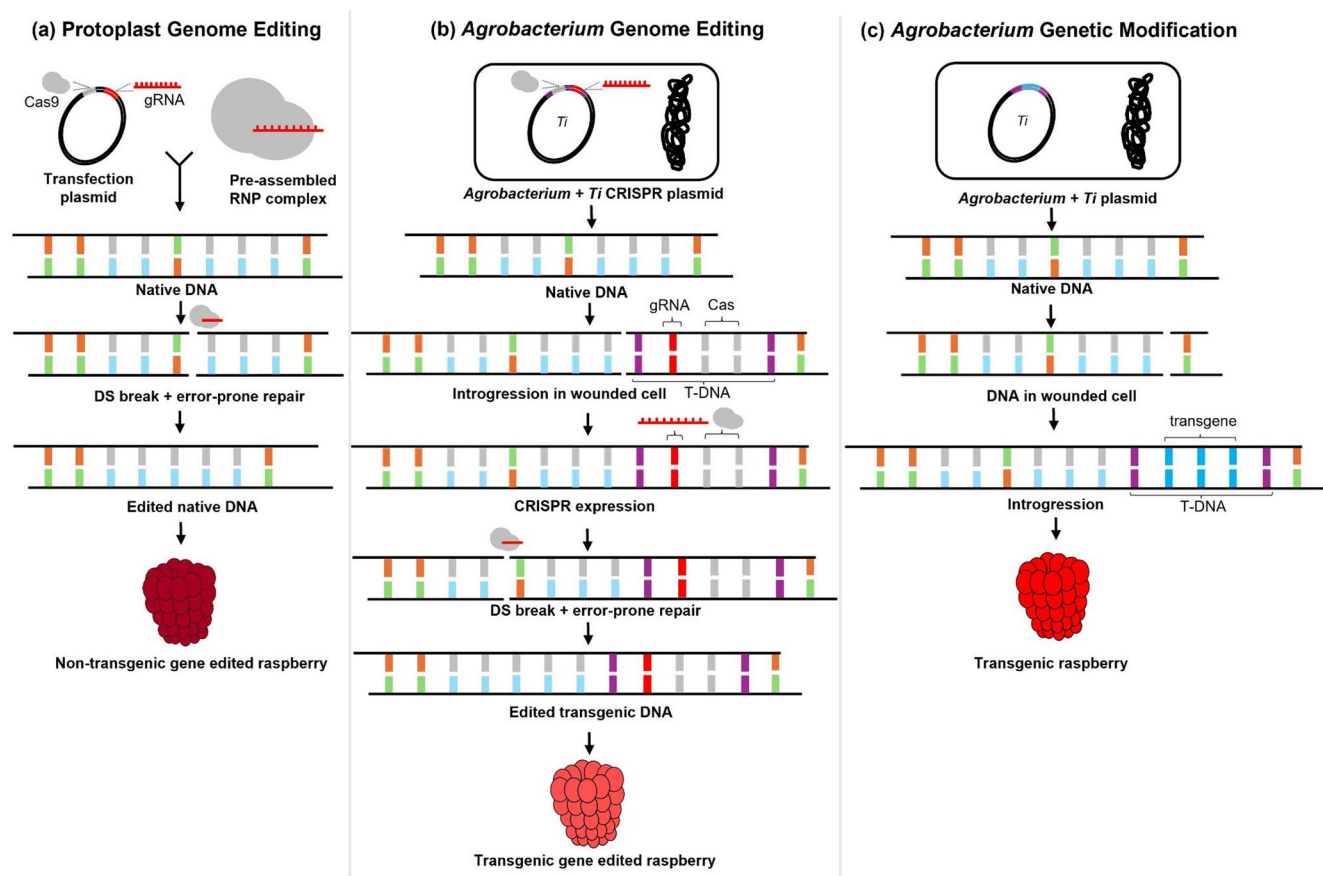


Fig. 2 Simplified diagram comparing DNA mutagenesis and transgenic status of three approaches for plant genome engineering in raspberry. **(a)** Non-transgenic protoplast transfection with plasmids containing RNP sequence or pre-assembled RNPs. RNPs initiate editing at the specific target site without introgression of foreign DNA and a non-transgenic gene edited raspberry can be recovered. **(b)** *Agrobacterium*-mediated genome editing introgresses T-DNA containing RNP sequences into a random genomic location. RNP components are expressed and initiate editing at the specific target site. The targeted

indels produced by protoplast or *Agrobacterium* genome editing are theoretically identical (e.g., –1 bp), but transgenes cannot be removed in raspberry through backcrossing without compromising the elite phenotype. Therefore, *Agrobacterium* genome editing would result in a transgenic gene edited raspberry. **(c)** Classic genetic modification via *Agrobacterium* transformation introgresses T-DNA containing a gene of interest into a random genomic location, resulting in a transgenic raspberry

cells, decoupling cell membranes from cell walls through dehydration, followed by an enzymolysis stage (Yue et al. 2021). Innovation in the field has improved protoplast yield, reduced damage caused by extraction and streamlined protocols. Variables including plant growing conditions, plant age, source organ, use of single cell culture, enzyme types, enzyme ratios, length of enzymolysis, basal media, hormones and supplements can all have dramatic effects on protoplast yield (Yue et al. 2021). Various plant tissues can be used for protoplast extraction, including flowers, stems, roots, and seeds, though leaves remain most common (Reed and Bargmann 2021). The health and age of the source tissue can be the most important variable for protoplast yield (Creeth et al. 2025) and regeneration success; some properties of the original tissue are retained (Lin et al. 2014) and actively growing, high-health source material yields more protoplast and promotes rapid division during regeneration (Phan et al. 1997; Lee et al. 2008).

There has been some convergence upon common methods for protoplast isolation, though modification is still required on a species-specific basis. One such method was developed by the Sheen lab (Yoo et al. 2007) in *Arabidopsis thaliana*, using Cellulase R-10 and Macerozyme R-10 alongside vacuum infiltration to extract high yields of protoplast from cotyledonous leaf tissue ($\sim 1 \times 10^7$ protoplast g^{-1} of leaf). Other enzymes can also be used such as driselase (Phan et al. 1997) and cellulysin (Barceló et al. 2019). Vacuum infiltration forces the enzymolysis solution into intracellular spaces in leaf tissue to improve enzyme penetration into the tissue (Chincinska 2021).

R. idaeus is a vegetatively propagated woody species, which makes protoplast isolation complex. Growing leaf material from seed is a common method to produce young, soft and sterile material such as cotyledons that can be easily enzymatically-digested (Yoo et al. 2007; Jeong et al. 2021) however the use of cotyledons is not appropriate in raspberry due to their derivation from seed. We recently shared work to mitigate this, isolating *R. idaeus* protoplast from young plantlets derived from vegetative stem cultures (Creeth et al. 2025). These plantlets were sufficiently soft that they could be digested with standard enzymolysis reagents like Cellulase R-10 and Macerozyme R-10 (Fig. 3). Protoplasts have also been isolated from raspberry using cell suspensions (Phan et al. 1997), circumventing the need for cotyledons as protoplast source but this does require continuous management and technical expertise.

Callus presents an alternative protoplast source, the first to form being non-friable callus, which is hard, green and slow growing. Protoplast isolation is possible from non-friable raspberry callus but results in low protoplast yields (Fig. 4b, e). Repeat subculture of non-friable calli over several months can form friable calli, which is off-white

to cream in colour, very soft, wet and grows rapidly. This material is highly amenable to protoplast isolation (Fig. 4c, f) as it disperses readily in solution and the rapid division rate may be beneficial during regeneration. Additionally, friable calli can be macerated, filtered and continually agitated through orbital shaking to generate single cell suspension cultures (Phan et al. 1997; Moscatiello et al. 2013). The use of flowers/reproductive tissue as explants can initiate embryogenic callus (EC) formation (Kang et al. 2020), identified through its ability to produce somatic embryos. To our knowledge EC has not been used for protoplast isolation in raspberry but EC-derived protoplasts in other species have been demonstrated to culture well and have good regeneration potential (Kang et al. 2020; Zhang et al. 2022b; Tricoli and Debernardi 2024). EC presents an attractive solution to regeneration recalcitrance in raspberry, however as a mostly biennial flowering species, generating reproductive material requires long-term planning and the elucidation of methods to produce EC in raspberry.

Protoplast transfection

A major advantage of protoplast-based methods is the relative simplicity with which genome editing can be performed. Once a high-quality isolate is available, protoplasts are highly permeable to genome editing components (such as RNPs), which can support high editing efficiencies. Reported indel frequencies from optimised protoplast transfection protocols are up to 90% in *Arabidopsis* (Jiang et al. 2021), 100% in oil palm (Norfaezah et al. 2024), 84% in chicory (Cankar et al. 2021), 60% in strawberry (Gou et al. 2020), 25% in cabbage (Murovec et al. 2018), and 19% in grapevine (Scintilla et al. 2022). However, a meta review found the average frequencies for protoplast-based transfection with polyethylene glycol (PEG) across all tested species are $\sim 17\%$ with wide range between different cultivars and species (Rustgi et al. 2022).

Protoplasts are transfected with either plasmids or pre-assembled RNPs, in tandem with PEG to increase membrane permeability (Yoo et al. 2007; Woo et al. 2015; Brandt et al. 2020; Lin et al. 2022). The protocol is straightforward: freshly isolated protoplasts ($1 \times 10^5 - 1 \times 10^6$) are mixed with either plasmids or RNPs in a 40% PEG4000 solution for 10–15 min (Yu et al. 2016). Protoplast are ashed via centrifugation to remove the cytotoxic PEG and incubated for 24–48 h, after which gene editing efficiency can be determined through DNA extraction and sequencing (Creeth et al. 2025), or protoplasts can be regenerated. Plasmids may permit more control over Cas9 and gRNA expression within the protoplasts, however random introgression remains a serious concern (Woo et al. 2015). The lack of DNA in pre-assembled RNP transfection eliminates

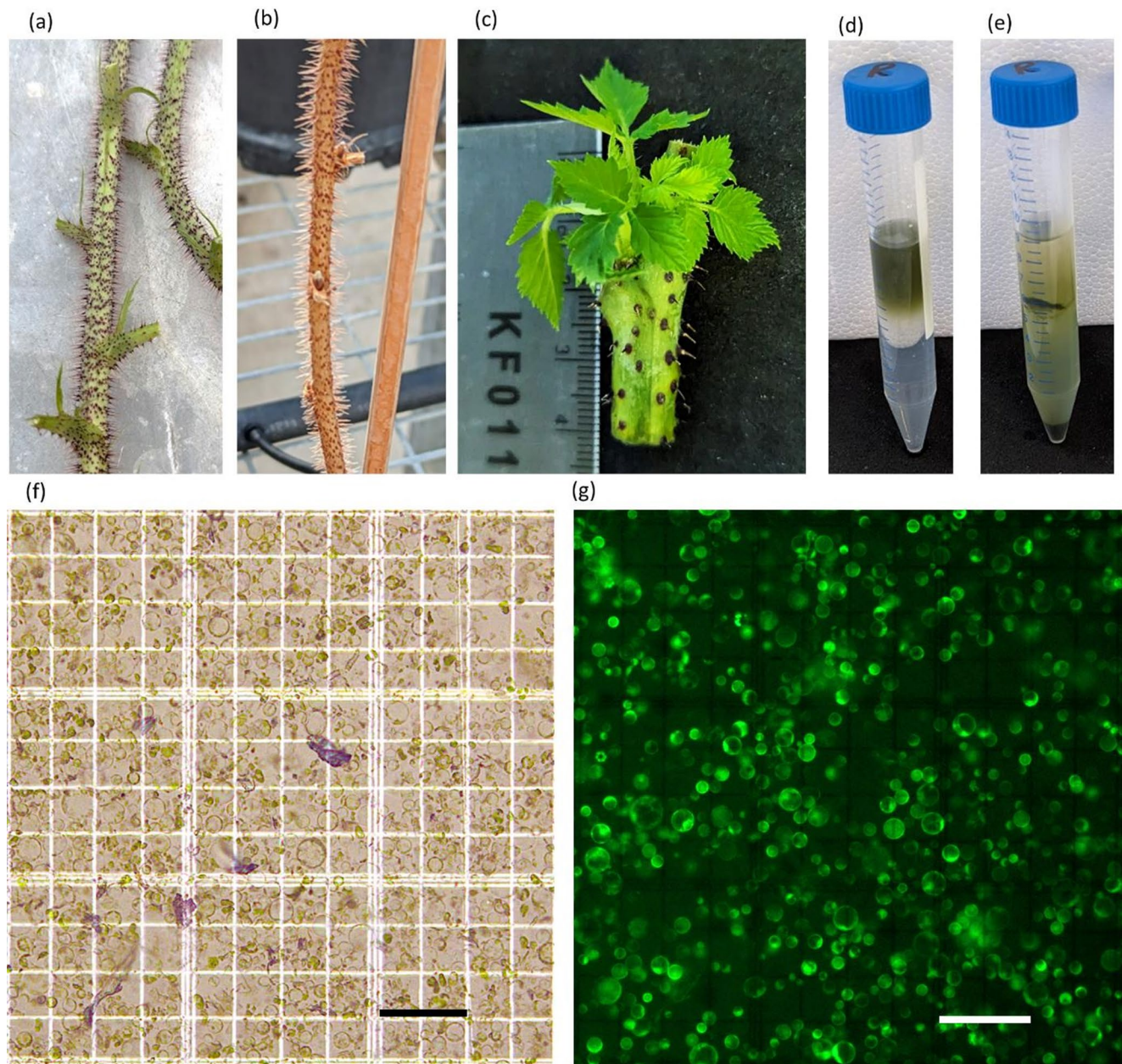


Fig. 3 Key steps for protoplast isolation in raspberry. **(a)** High-quality canes used for shoot culture, **(b)** low-quality cane, **(c)** young plantlets formed from shoot cultures, **(d, e)** purification of protoplast before **(d)**

and after **(e)** sucrose cushion, **(f, g)** protoplast isolated from plantlets under bright-field **(f)** and fluorescent **(g)** illumination (bars = 100 μ m). Reproduced from Creeth et al. (2025)

this concern. Pre-assembled RNPs are rapidly degraded by normal cellular processes, thus reducing the risk of off-target effects compared to plasmids with continuous RNP expression (Woo et al. 2015). RNPs have also been reported to result in significantly higher editing efficiencies than plasmid transfection (Jiang et al. 2021; Zhang et al. 2022b) and can be economically validated through in vitro cleavage tests (Brandt et al. 2020). Different Cas enzymes can also affect editing efficiency – there is evidence that Cas12a may result in higher editing efficiencies than Cas9 (Liu et

al. 2020; Zhang et al. 2022b). It is also simpler to use different gRNAs and Cas combinations with RNPs without the molecular cloning required for each new construct. We recently communicated new results where *R. idaeus* cult. BWP102 protoplasts were transfected with two synthetic gRNAs that targeted *phytoene desaturase* (*PDS*) at two intragenic loci. RNP transfection resulted in an editing efficiency of 19% and 2.3% respectively (Creeth et al. 2025). This proof-of-concept study established the feasibility of

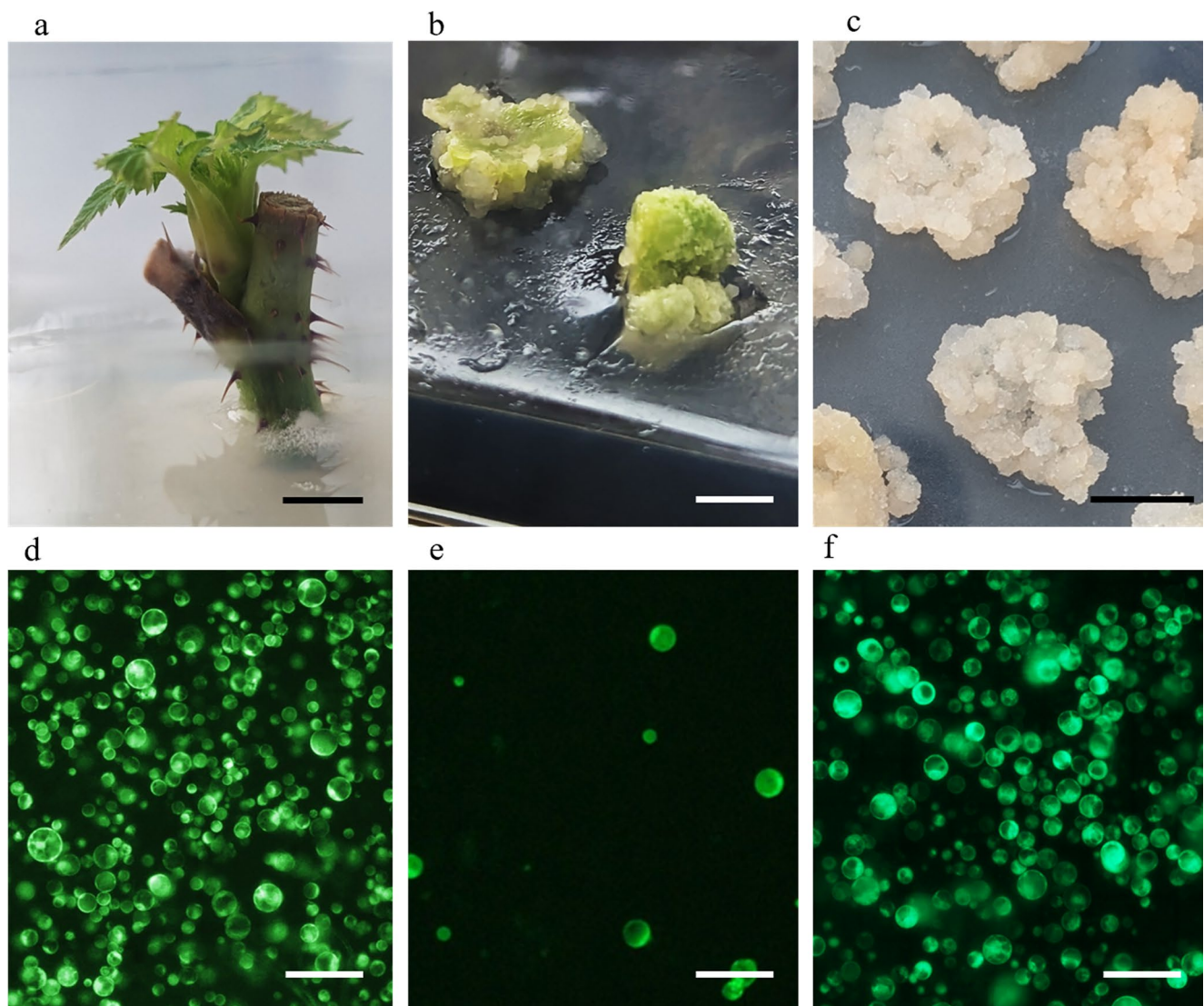


Fig. 4 Various potential source tissue for protoplast isolation in red raspberry, using protoplast isolation method outlined in Creeth et al. (2025); (a) stem culture plantlet, (b) non-friable callus, (c) friable cal-

lus, scale bars = 1 cm. Protoplast isolated from stem culture plantlets (d), non-friable callus (e) and friable callus (f), stained with fluorescein diacetate viewed under fluorescent microscope. Scale bars = 100 μ m

DNA-free editing in red raspberry and its effectiveness at inducing targeted mutagenesis.

Quantification of genome editing efficiency in protoplasts can be achieved several ways. The most accurate and comprehensive method is amplicon next generation sequencing (NGS) of target regions. Relative frequency analysis of the sequence pool can then be used to identify specific indels and their abundance in the pool at the target site (Huang et al. 2022; Geneious Prime 2024). Although costs are decreasing, NGS can still be cost-prohibitive, and a cheaper method of relatively accurately screening edits is through deconvolution of Sanger sequencing data. Deconvolution algorithmically aligns a gene edited sequence to a wild-type sequence and compares the base pair frequency in the gRNA binding region. A decrease in basepair confidence

is indicative of indels, and the algorithm estimates what indels are present and their frequencies. The programmes are free to use, and Sanger sequencing is substantially cheaper than NGS per sample (Brinkman et al. 2014; Bloh et al. 2021; Conant et al. 2022). We have found deconvolution to be broadly accurate compared to NGS (Creeth et al. 2025), however different programmes can produce different editing estimates for the same sample and struggle to identify insertions and larger deletions (Huang et al. 2022). The other technique of note is enzymatic denaturation (e.g., T7EI and Authenticase[®] assays). Gene edited PCR product is denatured into single-stranded DNA and re-annealed at low temperatures to create double-stranded amplicons containing an edited and WT strand. A mismatch-specific endonuclease then cuts the DNA at the indel site, with resulting

cleavage products visible via gel electrophoresis. Enzymatic denaturation is cheap and rapid but does not enable precise editing quantification and is not sensitive to low-frequency indels (Creeth et al. 2025). It is also difficult to reproduce accurately and can produce false positives (Germini et al. 2018). Rapid detection of gene editing can be also achieved by using two closely located gRNAs (Do et al. 2019) to create a large deletion, but this does not quantify the various indels in the population.

Protoplast regeneration

Compared to isolation and transfection methods, there is little consensus on protoplast regeneration. There is vast methodological variation in protoplast culture, indirect organogenesis and somatic embryogenesis between different species. Each stage of regeneration requires different media, hormones and environmental conditions and these in turn vary between species and varieties.

Many methods utilise hydrogels like sodium alginate as gelling agents for embedding protoplast (Jiang et al. 2021; Scintilla et al. 2022; Tricoli and Debernardi 2024). Unlike agarose (Barceló et al. 2019), alginate sets in the presence of Calcium ions rather than through heating and cooling. Gelling can thus occur at room temperature, which reduces the risk to protoplasts viability. Hydrogels provide support to protoplasts, preventing agglutination and permitting movement of nutrients and hormones to the cells. It is also easier to recover non-chimeric regenerants if the original single-celled protoplast are kept separate within a hydrogel and embedded protoplast can also be repeatedly studied as they progress through regeneration. Protoplast density within the gel is a critical factor for successful division, with some convergence on 1×10^5 protoplast mL^{-1} (Adedeji et al. 2020; Moon et al. 2021) but can also be as high as 1×10^6 protoplast mL^{-1} (Jeong et al. 2021). Alginate-embedded protoplasts are added to a liquid culture media and incubated in the dark for a couple of months while they divide into microcalli (cell colonies 100–500 μM in size). Alternatives to alginate include low-gelling temperature agarose (Park et al. 2019), which can be effective but is expensive, or liquid culture (Hsu et al. 2019; Lin et al. 2022). Liquid culture methods tend to result in protoplast agglutination and cell death, but can be effective for a minority of species, such as tomato (Lin et al. 2022) and tobacco (Lin et al. 2018).

The composition of culture media is a crucial factor in protoplast regeneration and is highly cultivar- and species-specific. A nutrient base is often used (e.g., Murashige and Skoog (Murashige and Skoog 1962), Kao and Michayluk (Kao and Michayluk 1974), Woody Plant Media (Tricoli and Debernardi 2024), alongside hormones and plant growth regulators (PGRs). A high auxin: cytokinin ratio

is commonly used for protoplast culture but is variable between methods. Some form of osmoticum (e.g., mannitol) is required to prevent protoplast membranes bursting and a carbon source (e.g., sucrose or glucose) is required as a food source. Supplementary single cell cultures can be used as feeder cells within the liquid media to produce conditioned medium and their use has been found to substantially improve regeneration success (Phan et al. 1997; Tricoli and Debernardi 2024). It is unknown what growth factors single cell cultures provide to encourage protoplast division, but it is hypothesised that they produce endogenous PGRs, growth peptides (Matsubayashi et al. 1996) and help to metabolise waste. Once protoplast re-grow cell walls, the osmoticum can be reduced over time to stimulate continuous division (Davey et al. 2005; Andreasson et al. 2022; Tricoli and Debernardi 2024). Regular subculture of the culture medium removes metabolic waste and refreshes PGRs, supporting continuous division of protoplasts into microcolonies and microcalli.

Microcalli (e.g., 0.5–1 mm diameter) are then added to solid media to form true calli. Media is usually set with agar or Gelrite™ and often contains the same nutrient base as the prior liquid culture media. Microcalli are exposed to light ($30\text{--}50 \mu\text{mol m}^{-2} \text{s}^{-1}$) to trigger further callus proliferation (Lin et al. 2022). Depending on the protoplast source, calli undergo somatic embryogenesis (Kang et al. 2020) or indirect organogenesis (Scintilla et al. 2022). A high cytokinin: auxin ratio is normally required at this stage to stimulate shoot production but is a critical variable for optimisation. In vitro shooting is a significant challenge when developing novel regeneration methods as some species/cultivars are fundamentally recalcitrant (Roubelakis-Angelakis 1993). Reporter genes such as *phytoene desaturase* (*PDS*) are useful at this stage as any regenerated shoots from a null mutant will display a distinctive albino phenotype (Wilson et al. 2019).

The type of material from which the protoplast derive is a crucial factor. While leaf-derived protoplasts can undergo indirect organogenesis in some species, for some species leaf protoplasts are recalcitrant to regeneration. EC offers an alternative protoplast source, particularly for woody species (Kang et al. 2020; Zhang et al. 2022b; Tricoli and Debernardi 2024), that has been shown to be conducive to somatic embryogenesis. It is unknown whether EC can be generated in raspberry, however exploration and assessment of all available protoplast sources is essential to identify suitable options for regeneration.

If shooting can be achieved, the remainder of the regeneration process is more straightforward. The shoots are cultured on the callus until large enough to be excised ($> 1 \text{ cm}$) and added to either auxin media or media lacking hormones to stimulate rooting (Jiang et al. 2021). Once rooting occurs,

the shoot grows into a full plant (Fig. 5). Optimised protoplast regeneration with DNA-free genome editing can produce a plant with a genetically improved phenotype within six months (Najafi et al. 2023). For example, DNA-free editing followed by protoplast regeneration has been used for improved downy and powdery mildew resistance in grapevine (Tricoli and Debernardi 2024), flowering repression

in tomato (Liu et al. 2022) and glucosinolate reduction in rapeseed (Li et al. 2021b).

There are genetic and physiological constraints that hamper raspberry protoplast regeneration compared to model species where regeneration is more straightforward. Raspberry, like other Rosaceae, produces many phenolic compounds in tissue culture which prevents straightforward protoplast culture (Ahmad et al. 2013; Xu and Wang 2025).

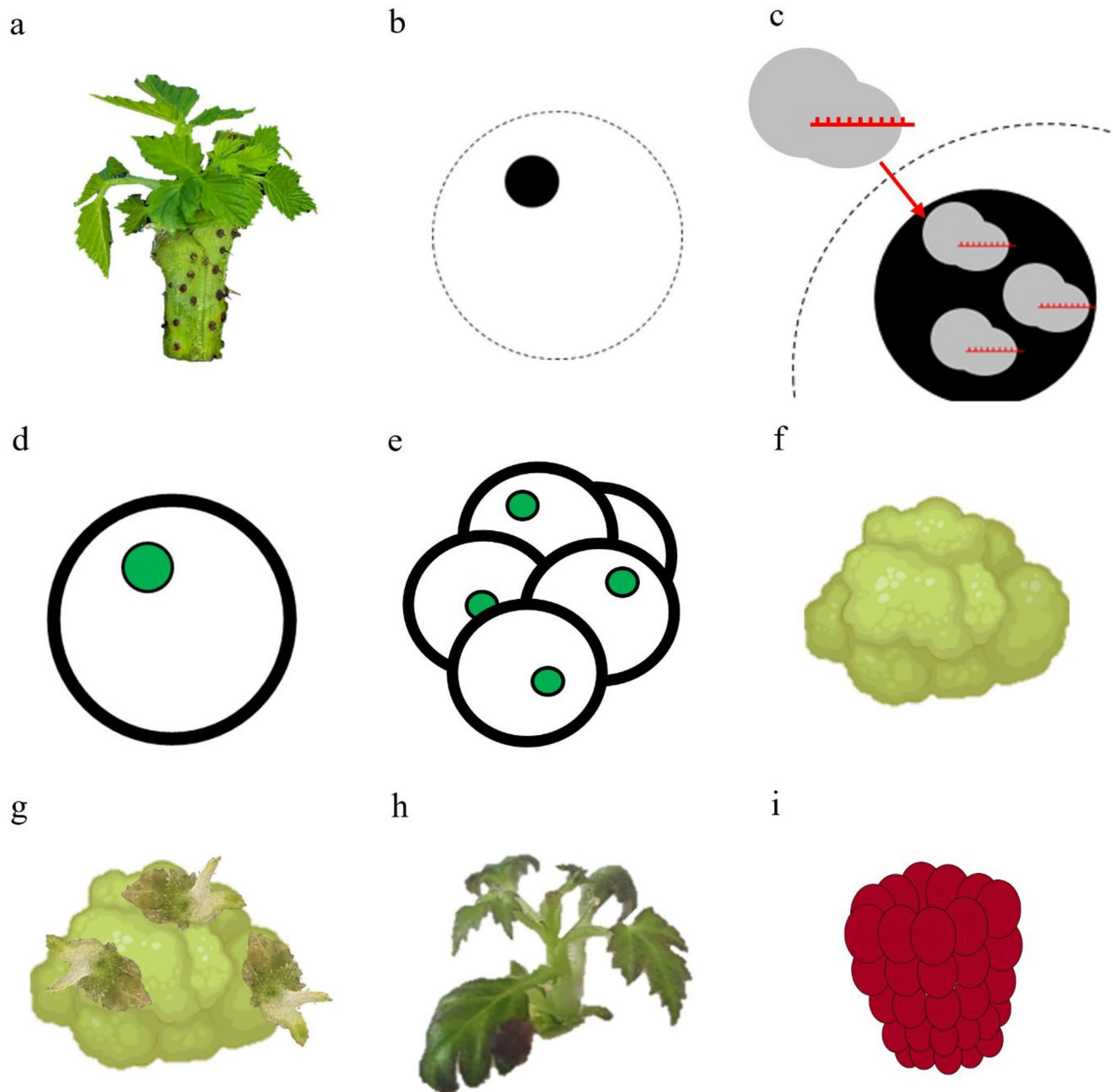


Fig. 5 Overview of DNA free genome editing process in raspberry. (a) Stem cultured plantlet used as protoplast source, (b) isolated protoplast, (c) protoplast transfection with pre-assembled RNPs, (d) gene edited cell with regrown cell wall, (e) culture of gene edited cells into

a microcolony, (f) regenerated callus, (g) indirect organogenesis on regenerated callus, (h) excised regenerated plant (i) gene edited raspberry obtained from regenerated plant. Panels (f) and (g) use assets from BioRender

Furthermore, compared to species like *Arabidopsis* or tomato, there are no optimised supporting protocols available for protoplast isolation, gene editing, protoplast culture or embryogenesis/organogenesis. Tissue culture remains more of an onerous art than a science primarily because of a lack of understanding of interspecific differences; this is exacerbated in species like red raspberry with few high-quality protocols for even in vitro micropropagation. This is in contrast to fruit species like strawberry (Barceló et al. 2019), grapevine (Scintilla et al. 2022; Najafi et al. 2023; Tricoli and Debernardi 2024), apple (Perales and Schieder 1993) and citrus (Soriano et al. 2022) where existing protocols for regeneration are available (Ma et al. 2023). Red raspberry protoplasts have been only once cultured to microcalli in a small study that used single cell suspensions of raspberry as a protoplast source (Phan et al. 1997). Protoplast were cultured in KM8p media (Glimelius et al. 1986) and a range of supplementary PGRs and feeder cells were tested to analyse regeneration efficiency (Phan et al. 1997), however shoots could not be recovered from regenerated calli. This leaves a substantial research gap in protoplast regeneration in red raspberry.

In the absence of further literature specific to *R. idaeus* protoplast regeneration, the body of work of *R. idaeus* micropropagation in tissue culture offers an alternative source of information, as the technique requires stimulation of in vitro shoots from leaf-derived callus. Kálai et al. (2009) found pre-treatment of leaves in the dark for up to a week on MS media with B5 vitamins containing the cytokinins 6-benzyl aminopurine (BAP) and thidiazuron (TDZ) increased regeneration rates 4–23 x depending on the variety. The optimal shoot regeneration media was MS salts with B5 vitamins, supplemented with a high concentration of BAP and low concentrations of TDZ and indole-3-butyric acid (IBA). Significantly, all six *R. idaeus* cultivars tested could be regenerated using this media. McNicol and Graham (1990) succeeded in obtaining shoots from both leaf and stem segments. Media type was influential and found that optimal PGRs were dependent on the culture media used. For both leaf discs and stem segments, cultivar greatly affected regeneration efficiency. Activated charcoal, commonly used in protoplast regeneration methods, was found to totally inhibit organogenesis. BAP and IBA was a successful PGR formulation for both tissue types, whereas zeatin and 2,4-D, BAP and 2,4-dichlorophenoxyacetic acid (2,4-D) were only suitable for regeneration on stem segments. This study and other literature (Phan et al. 1997; Mezzetti et al. 1999; Kálai et al. 2009) identify BAP as a key cytokinin in *R. idaeus* tissue culture, while choice of auxin is more variable. However, the same group later found TDZ at high levels and NAA at very low levels to be a more effective PGR combination than BAP and IBA (Graham et

al. 1997). TDZ and NAA were also found to be an optimal combination in a separate study (Turk et al. 1994). There is a need for comprehensive research on PGR types and combinations to understand which are most optimal for in vitro shooting in a wide range of red raspberry cultivars.

The evidence of regeneration potential in *R. idaeus* lends credence to the feasibility of protoplast regeneration in this species; it is proven raspberry callus can be induced to shoot, thus it is theoretically possible to achieve indirect organogenesis from protoplast-derived calli. However, red raspberry remains a challenging woody species and in vitro shooting may only have limited applicability to protoplast regeneration (Perales and Schieder 1993; Hassanein and Dorion 2006; Chupeau et al. 2013; Ikeuchi et al. 2019; Jiang et al. 2021).

Remaining challenges

Protoplast source

Despite recent progress that demonstrates the feasibility of DNA-free genome editing in raspberry protoplasts, substantial challenges remain before it can be commercially exploited. Isolating protoplast from stem cultures can result in inconsistent yields of protoplast and are strongly dependent on original cane health (Creeth et al. 2025). The short timescale between incubating stems in tissue culture and harvest of plantlets (10 days) also increases the risk of microbial contamination (Creeth et al. 2025). While current enzymes and their concentrations produce sufficient protoplast, the leaf tissue as a whole remains intact rather than fully disintegrating (Yoo et al. 2007), suggesting many more protoplast (particularly of mesophyll origin) could be isolated with a more optimised protocol, such as those available in species like *Arabidopsis* (Jeong et al. 2021). Alternatively, the plantlets used as a protoplast source could be grown into larger plants and then split into fully in-vitro stem cuttings. A second generation of plantlets could then be grown and used as a protoplast source – one step removing the protoplast source material from the sterilisation process to reduce the chance of microbial contamination. Anecdotally, the longer raspberry plants are micropropagated in tissue culture, the lower the protoplast yield, suggesting the raspberry plant tissue culture process requires optimisation to maintain very high plant health. Consequently, the current recommended time in tissue culture for raspberry stem cultures is 10 days (Creeth et al. 2025). Protoplast derived from calli are much less susceptible to contamination, however the development of friable callus and EC is not well studied in raspberry. Callus must be extremely friable and dissipate readily in solution for good enzymolysis and high

protoplast yields. Isolating protoplast from cell suspensions requires both technical expertise to cultivate the suspensions and continuous subculture of cell stocks. For both calli and cell suspensions, extended time in tissue culture before regeneration may also be undesirable as it can increase the prevalence of random somatic mutations and cause a loss of shooting potential (Wang and Wang 2012).

Transfection efficiency

While we managed to attain a transfection efficiency of up to 19.0% in raspberry protoplasts, four other gRNAs tested achieved editing efficiencies between 0.3 and 6.0% (Creeth et al. 2025), demonstrating substantial variation. Factors including targeted genomic region (promoters/exons), GC content, relative composition of purines and pyrimidines, gRNA length and gRNA secondary structure have all been shown to effect gRNA activity and have been comprehensively reviewed elsewhere (Javaid and Choi 2021). gRNA design algorithms have been an valuable development in this space considering the breadth of factors to consider (Doench et al. 2014). It can also be beneficial to use multiple gRNAs against the same target gene to boost editing efficiency (Armario Najera et al. 2019), however this has not yet been attempted in raspberry. Editing efficiency is known to be variable between gRNAs irrespective of plant species, although greater challenges with transfection/transformation of woody plants are acknowledged compared to model plants (Osakabe et al. 2016). Many other aspects of transfection can also be optimised: quantities and molar ratios of RNP components can be anywhere in the range of 5 µg to 50 µg, with 1:1 (Cankar et al. 2021), 2:1 (Park et al. 2019) and 1:2 (Norfaezah et al. 2024) ratios of Cas9: gRNA common. The incubation time of RNPs with protoplasts generally varies between 24 and 48 h (Brandt et al. 2020; Norfaezah et al. 2024) though PEG-transfection is usually limited to 10–15 min due to PEG cytotoxicity. The concentration of PEG4000 has been explored as a target for optimisation of transfection, however a concentration of 40% has consistently been found to result in the highest transfection efficiency in multiple species (González et al. 2020; Wang et al. 2022) but can reduce viability. Cycles of regeneration take longer for slow growing species, which makes genome editing research complex in woody species. The result in red raspberry is limited availability of methods (in particular for tissue culture (Mathews et al. 1995; Phan et al. 1997; Mezzetti et al. 2004b) which can affect attempts to optimise transfection efficiency (Osakabe et al. 2016; Creeth et al. 2025).

Validation of gRNAs in vitro is an important precursor to establishing successful in vivo transfection, with a protocol now outlined in raspberry (Creeth et al. 2025). In vitro

cleavage tests challenge PCR product containing the target loci against small quantities of RNPs (Brandt et al. 2020) to evaluate gRNA sequence complementarity but can be complex to scale up. High-throughput screening of gRNAs has also been a key focus of research in recent years to identify effective gRNAs through brute force (Pan et al. 2023). Libraries of gRNAs are created against the whole genome and screened simultaneously as a pool (CRISPR-based pooled screens). Large scale DNA barcoding and next generation sequencing is then used to determine the most effective candidate gRNAs. Applying such a system to raspberry breeding would require a level of funding not yet available but has been applied successfully at scale in species such as rice (Lu et al. 2017). Researchers created a library of 88,500 gRNAs targeting 34,000 genes, with an average mutation rate of 83.9% in 84,000 regenerated plants (Lu et al. 2017). Transfection efficiency also needs to be high (e.g., >20%) because protoplast regeneration does not involve selection, and one study in *Arabidopsis* estimated that only 0.5% of protoplast divide into microcalli (Xu et al. 2021).

Plant recovery from regenerated callus

Protoplast regeneration remains the greatest challenge with DNA-free genome editing in any prospective species, but particularly in red raspberry. Successful plant recovery requires several optimised methods chronologically threaded together: protoplast isolation, transfection, protoplast culture, microcallus culture, *de novo* shoot regeneration and plant acclimation. Protoplast regeneration inherently needs adaptation to each new species due to the sensitive dynamics of culturing single cells into whole plants. This hampers rapid development of DNA-free techniques in non-model species and requires specific technical expertise in protoplast tissue culture. Protoplasts must be physically supported, at an appropriate density, supplied with custom nutrients and hormones while removing waste products. Osmoticum must be reduced over time to stimulate continuous division, and regenerated microcalli have to survive movement from liquid to solid culture without inducing excessive stress. There are a huge number of variables throughout the process to be considered and optimised, which inescapably takes years of method development.

Regeneration methods have advanced and become optimised in recent times in model species, particularly *Arabidopsis* (Yoo et al. 2007; Wu et al. 2009; Chupeau et al. 2013; Jeong et al. 2021; Tan et al. 2023), tobacco (Hsu et al. 2019, 2021), tomato (Lin et al. 2022; Liu et al. 2022) and potato (González et al. 2020; Moon et al. 2021). A broader focus is required on underserved, yet agronomically important, crop species to enable the application of next generation breeding technologies across the spectrum of crop species. There

have been several reviews (Eeckhaut et al. 2013; Reed and Bargmann 2021; Mukundan et al. 2025) that will help such endeavours, though we also encourage the development of precision breeding-focused events and workshops to improve links and knowledge-sharing within the community of breeders, growers and researchers. When developing protoplast regeneration methods for new species, *de novo* callus shooting e.g. for *Agrobacterium* transformation, may offer practical insights on suitable in vitro conditions. However, there is a broad evidence base that protoplast regeneration is contingent upon different molecular processes to callus regeneration (Perales and Schieder 1993; Hassanein and Dorion 2006; Chupeau et al. 2013; Ikeuchi et al. 2019; Jiang et al. 2021). This limits the bearing of genetic modification and in vitro shooting research (Mathews et al. 1995; Mezzetti et al. 2004a; Popescu 2009) on DNA-free genome editing in red raspberry.

Broader challenges of DNA-free genome editing in crop species

While many published studies have achieved DNA-free genome editing through protoplast regeneration, critical experimental information is not always included – particularly with regard to regeneration methods. Protocol details such as light intensities, day: night cycles, subculture frequencies, protoplast culture densities are essential for experimental replicability, but are not consistently available in some literature (Phan et al. 1997; Lin et al. 2018; Tricoli and Debernardi 2024). While this can be due to publication stringency (e.g., word counts) and the inherent complexity of protoplast regeneration, there is also increasing commercial interest in fully functional protoplast regeneration methods which could incentivise the protection of critical protocol details. Patenting of methods is consequently widespread (Kral and Pelton 2022; Tricoli 2022) and there have been a number of spin-out and established companies entering the field of DNA-free genome editing. Commercialisation is an important and expected part of applied biotechnology research, however if important results are not publicly shared or poorly replicable, it may damage the positive feedback loop of publicly available research leading to new funding (Fig. 6). This may also stymie innovation and “cross-pollination” within the precision breeding community. The perception of the over-industrialisation of GM foods was instrumental in public opinion turning against an otherwise socially, economically and environmentally beneficial biotechnology (Durant and Lindsey 2000; Lang and Hallman 2005). For example, recent innovations by a US commercial setup in gene edited blackberries and black raspberries (Souder 2024) are advertised as non-transgenic; but it is unclear how this was achieved (Aubin et al. 2025).

Such protocols would be of great interest to the raspberry breeding community. A careful balance must therefore be struck between rewarding innovation through intellectual property protection while ensuring the accessibility of innovation to the wider research community.

One key challenge for plant genome editing as a field is ensuring continued convergence of artificial intelligence (AI) with genome editing techniques (Li et al. 2025). AI models are particularly advantageous as they can be used holistically throughout the genome editing process. The use of “big data” in the plant sciences has grown exponentially over recent decades; convolutional neural networks (CNNs) and recurrent neural networks (RNNs) are becoming essential machine learning (ML) tools. They enable better understanding and analysis of data for the construction of gene regulatory networks and linking genomic, proteomic, transcriptomic datasets together. Genomic data analysis could also be improved by deep ML methods to detect and predict gene variants and their functions, which is essential when predicting how a specific targeted mutation would affect the crop phenotype. New research has yielded predictive CNNs like CRE.AI.TIVE (Jevtic et al. 2024) that predict the effect of mutagenesis in gene promoters and regulatory elements on gene expression; validation through massively parallel reporter assays can help identify optimal gRNAs and gRNA binding sites (Jores et al. 2021; Tan et al. 2023; Jevtic et al. 2024). Instead of repetitive and iterative laboratory trials for every possible permutation of gRNA, algorithms are regularly applied to identify the most effective and specific gRNAs for a given sequence (Doench et al. 2014; Liu et al. 2020) by combining custom genome sequence data with CNNs trained on historical gRNA efficacy. New paradigm-shifting tools in this space are announced regularly, such as AlphaGenome (Avsec et al. 2026). The continued improvement of editing efficiency using more recent ML-driven gRNA design (Qu et al. 2025) would be useful particularly for species without existing optimised gene editing methods like raspberry. Protein engineering for genome editing can be guided by AI, improving existing Cas enzymes (He et al. 2024) or even moving beyond them entirely (Huang et al. 2023). One central issue with genome editing is the lack of knowledge of actual gene function; even in *Arabidopsis*, only 74% of the genome is functionally understood (Reiser et al. 2024). ML is well suited to predict gene functions by using existing experimental data on genotype-phenotype linkage, gene expression analyses and loss-of-function mutants (Dixit et al. 2024), though much more plant-specific training data is required to improve the quality of predictions (Li et al. 2025). Scaling this to understand the complex genetic and environmental causes of agriculturally relevant polygenic traits would be a highly valuable application of AI to plant breeding. This is particularly relevant

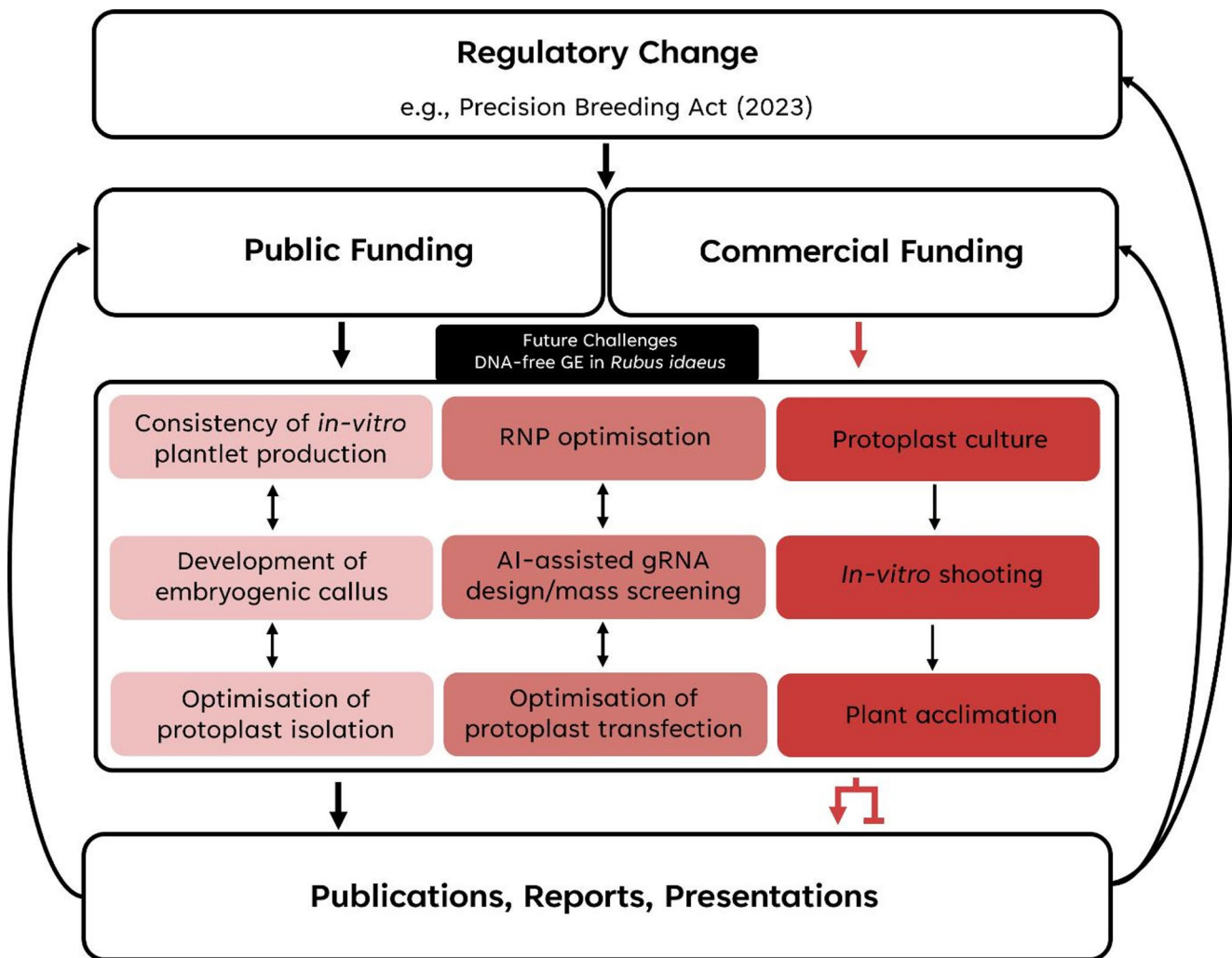


Fig. 6 Ecosystem relationships between drivers of research and remaining challenges in developing DNA-free genome editing in red raspberry. Regulatory change triggers greater interest in precision breeding research. This drives public and commercial funding, which is essential to solve the remaining challenges of DNA-free genome editing in raspberry. The scientific publication, review and dissemination of progress is crucial to stimulate further progress in this field and drive further regulatory change, however commercially-funded research is often intellectually protected and may not be disseminated to the research community, disrupting positive feedback loops in the ecosystem

in decoding the genetics and genomics of poorly understood species like red raspberry, as it eliminates the need for the replication of decades of experimental work in model species. Current strategies for identifying and targeting genes of interest, especially for non-model species, are a moonshot with no guarantee that the loss-of-function mutant will result in the desired phenotype. Applying cutting-edge ML approaches with genome editing would greatly contribute to the fundamental understanding of highly valuable traits like yield or firmness in *R. idaeus*.

tion of progress is crucial to stimulate further progress in this field and drive further regulatory change, however commercially-funded research is often intellectually protected and may not be disseminated to the research community, disrupting positive feedback loops in the ecosystem

Conclusion

Genome editing continues to develop at pace, with constant evolution of what is technically and experimentally possible. Protoplast regeneration offers an opportunity in red raspberry cultivation to shift the industry toward faster, more effective and more fruitful breeding. Recent progress with protoplast isolation and DNA-free genome editing demonstrates the feasibility of non-transgenic techniques in red raspberry, while regenerated plant recovery and successful genotype-phenotype linkage remain barriers to progress. More generally, improvements in method quality, consistency and openness are required while successful integration of new technologies will be critical to fully harvest the potential of genome editing in red raspberry.

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Data availability Data sharing is not applicable to this article as no datasets were generated or analysed during the current study.

Declarations

Ethics approval Not applicable.

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