AN ABSTRACT OF THE THESIS OF

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Title: Overexpression of the *GROWTH REGULATING FACTOR 4-GRF-INTERACTING FACTOR 1* Transcription Factor Chimera Modifies Transformation and Regeneration Efficiency in *Populus* and *Eucalyptus*

Abstract approved:

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Steven H. Strauss

Transformation is a major bottleneck for genetic engineering and gene editing in forest tree species. This includes most genotypes of *Populus* and *Eucalyptus*, which are some of the world's most widely-cultivated genera of plantation forest trees. To provide new tools for transformation, I tested the transcription factor-protein chimera consisting of *GROWTH REGULATING FACTOR 4 (GRF4) & GRF-INTERACTING FACTOR 1 (GIF1)*, which gave large increases in regeneration of transgenic shoots in citrus and other crop species. Using an *Agrobacterium* organogenic system, I tested various configurations of the chimera, including different levels of miRNA sensitivity and promoter strengths.

I transformed two hybrid poplar genotypes with binary vectors containing an empty vector control and either a 2x35S:miRNA-resistant GRF4-GIF1 chimera from *Citrus* provided by the Dubcovsky laboratory at the University of California at Davis, or a 1x35S:miRNA-sensitive GRF4-GIF1 chimera from *Populus* that I created. The poplar GRF4 and GIF1 genes were inferred from the *Populus* genome following a phylogenetic analysis comparing all *Populus* GRF and GIF proteins with those from *Citrus, Vitis*, *Eucalyptus,* and *Oryza*, and then I removed the GRF4 stop codon and fused it to a synthetically-derived linker with four amino acids (AAAA) and then to the GIF coding region. A red fluorescent protein-encoding gene was added to both constructs to enable rapid quantification of transgenic regeneration rates. The chimeric

GRF-GIF sequences were cloned into an expression vector and poplar as well as eucalypt tissue explants were transformed using *Agrobacterium.*

I found that the effects of overexpressing the *Citrus* miRNA-resistant GRF4-GIF1 chimera were highly genotype-specific. A significant negative regeneration phenotype was observed in a *P. tremula x tremuloides* genotype ('353-53') while a *P. tremula x alba* genotype ('717-1B4') had no significant difference in regeneration versus an empty vector control (mean reduction of 95% and 4%). The negative regeneration phenotype in 353-53 and the interaction between genotype and overall effect of *Citrus* GRF-GIF overexpression were statistically significant ($P < 0.01$). There was also a statistically significant decrease in ectopic root formation observed on leaf disc explants from 717-1B4 ($P < 0.01$), suggesting that *Citrus* GRF4-GIF1 overexpression may suppress rooting in poplar.

Results from overexpression of the *Populus* miRNA-sensitive GRF4-GIF1 chimera (*Pt*GRF-GIF) were less genotype-dependent. Transgenic shoot regeneration nearly doubled in genotype 717-1B4 over the empty vector control, but the effect did not reach statistical significance ($P = 0.07$). In genotype 353-53, there was no appreciable change in transformation rate nor did effects approach statistical significance. Interestingly, *Pt*GRF-GIF reduced transgenic callus formation in both genotypes (mean reduction of 65%), which was statistically significant in 353-53 ($P = 0.03$). All transgenic *Populus* GRF4-GIF1 shoots from genotype 353-53 died when placed on elongation medium (EM), but no 717-1B4 *Populus* GRF4-GIF1 shoots died. This difference in shoot viability between genotypes is statistically significant (Fisher's exact test, $P = 0.02$).

Non-replicated exploratory experiments with additional GRF-GIF chimeras suggested that GRF4-GIF1 transcript stability (miRNA396-resistance) level was inversely correlated with transgenic shoot regeneration rate. Constructs which were mutated to be miRNA-resistant consistently reduced shoot regeneration in two hybrid poplar genotypes more than a miRNA-sensitive construct from *Citrus*. Additionally, a dexamethasone-activated miRNA-resistant chimera from *Vitis* reduced shoot regeneration in both poplar genotypes when dexamethasone was applied. A non-replicated experiment which tested the *Populus* GRF4-GIF1 in a recalcitrant *P. alba* genotype ('6K10') resulted in a high transgenic shoot regeneration rate (37%), compared to a complete lack of regeneration in an empty vector control (Appendix Figures C.4 & C.5).

Some GRF-GIF constructs were also tested in hybrid eucalypt, however many of the experiments suffered from poor transformation efficiency, tissue necrosis, and contamination. Overexpression of the *Citrus* miRNA-resistant GRF4-GIF1 chimera in two hybrid eucalypt genotypes increased the prevalence of transgenic callus (mean increase of 35%), but the effect did not reach statistical significance in either genotype ($P = 0.12$ & 0.9). Additionally, a *Eucalyptus grandis* GRF5-GIF1 chimera which I produced had unclear effects on transformation and regeneration in a non-replicated experiment. The dexamethasone-activated miRNA-resistant chimera from *Vitis* increased the prevalence of transgenic callus and shoot primordia in two eucalypt genotypes (mean increase of 28%).

My results suggest that certain GRF-GIF chimeras may increase the regeneration rate of transgenic shoots in select genotypes of *Populus*, while having a negative impact on the root and callus development pathway in other genotypes. A larger study with more tree genotypes, GRF-GIF sources and miRNA sensitivities, and a wider array of promoter strengths and/or an inducible or excision system, may help shed light on the wider potential of GRF-GIF to promote regeneration of transgenic poplars and eucalypts.

Overexpression of the *GROWTH REGULATING FACTOR 4-GRF-INTERACTING FACTOR 1* Transcription Factor Chimera Modifies Transformation and Regeneration Efficiency in *Populus* and *Eucalyptus*

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I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

Nathaniel W. Ryan, Author

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CONTRIBUTION OF AUTHORS

Cathleen Ma helped perform the transformation and propagation of plant material and provided technical expertise in all aspects of plant tissue culture, cocultivation with *Agrobacterium*, regeneration/selection, and analysis of transgenic tissues. Dr. Greg Goralogia cloned the *Citrus* and *Vitis* GRF-GIF genes into expression vectors and provided primer design and cloning expertise for cloning of the *Populus* and *Eucalyptus* genes. Michael Nagle wrote the GMOdetector software code that enabled high-throughput analysis of transgenic callus. Dr. Zander Myburg and Bernard Smit helped conduct the phylogenetic analysis that led to the cloning of GRF and GIF genes. Dr. Steven H. Strauss oversaw all research performed in this project.

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TABLE OF CONTENTS

Page

TABLE OF CONTENTS (Continued)

Page

TABLE OF CONTENTS (Continued)

Page

LIST OF FIGURES

Figure Page

LIST OF FIGURES (Continued)

Figure Page

LIST OF TABLES

Table Page

LIST OF APPENDIX FIGURES

Figure Page

LIST OF APPENDIX FIGURES (Continued)

Figure Page

LIST OF APPENDIX TABLES

Chapter 1: Scientific Background

1.1 Scope and Organization of Thesis

1.1.1 General research goals

In this thesis I first provide a literature review of relevant subject matter, then describe my work, and that of collaborating lab members, to explore the potential value of GRF-GIF to poplar regeneration and transformation. Much of the early experiments were highly exploratory, as I learned *in vitro* and transformation methods and sought to test variety of genotypes and construct types. The experiments that I chose to replicate because of their results and promise are described in the main body of the thesis, while the non-replicated exploration experiments, including *Eucalyptus* transformations, are briefly documented in the appendix, and summarized in results section of the thesis.

Transformation and regeneration remain primary bottlenecks for the application of recombinant biotechnology to forest trees. Overexpressing and/or ectopically expressing developmental regulator genes (henceforth 'DEV' genes) is one possible approach to surmounting this obstacle. This research is part of a large, multi-year effort in our laboratory to develop methods which promote transformation and/or regeneration in a large number of *Populus* and *Eucalyptus* genotypes.

This larger project can be categorized into the following major areas:

a) Develop media conditions and transformation protocols which promote transformation and transgenic shoot formation
b) Identify DEV genes that promote transformation and/or regeneration of *Populus* and *Eucalyptus*
c) Identify promoters, including expression induction systems, which stimulate DEV genes in the proper spatio-temporal context for promotion of regeneration
d) Develop methods for DEV gene excision to avoid the pleiotropic effects of constitutive DEV gene expression beyond its window of usefulness

This document contains information pertaining to the methods and results from using a novel DEV gene for forest trees, namely a protein chimera consisting of *GROWTH-REGULATING FACTOR* (*GRF*) and *GRF-INTERACTING FACTOR* (*GIF*), in *Populus* and *Eucalyptus*.

1.2 The genus *Populus*

1.2.1 Distribution and botanical description of *Populus*

The genus *Populus* is a member of the Salicaceae family and contains 25-30 species of deciduous trees divided amongst six sections (Stettler & National Research Council of Canada, 2000). Known colloquially as 'poplars,' the genus is natively distributed throughout the Northern Hemisphere and contains the aspens and cottonwoods, as well as many interspecific hybrids (Isebrands & Richardson, 2013). Cottonwoods are often found growing in wetlands or in riparian zones, while aspens are considered one of the most important boreal angiosperm trees (Meikle & Gordon, 2001).

There are several identifying characteristics of *Populus*. On young trees, the bark is smooth (usually white or greenish) and often has conspicuous lenticels. In some species, older trees develop rough and deeply fissured bark, while others retain their smooth bark throughout their life. The leaves of *Populus* are arranged spirally, and range in shape from triangular to circular. *Populus* often has long petioles, which gives the tree a "twinkling" or "quaking" appearance in the wind. Additionally, the leaves often turn an exquisite bright yellow before falling during autumn (Meikle & Gordon, 2001; Rushforth, 1999).

Populus spp. are usually dioecious, with monecious flowers being considerably rarer. Flowering occurs in early spring before the leaves appear. Flowers are borne in drooping, sessile, or pedunculate catkins which form in the axils of the previous year's leaves. Pollen is dispersed by wind. Fruits are dehiscent capsules, and contain small seeds surrounded by soft white "cotton" which aids in wind dispersal (Keeler, 1900; Meikle & Gordon, 2001).

Since the 18th century, botanists and breeders have produced many interspecific hybrids between European and North American poplar species. Many poplar hybrids display heterosis, a.k.a. 'hybrid vigor,' and have been popular worldwide since their introduction (Henry, 1912; Stout & Schreiner, 1933). Poplars are commonly grown as ornamental trees due to their rapid growth, and species with fastigate branching are especially prized across Europe and Asia

(Rushforth, 1999). Today, poplars are also planted in commercial plantations on over 31 million total hectares in at least 24 countries (FAO & IPC, 2016).

Figure 1.1: Pistillate catkins of *Populus tremuloides* growing near Bozeman, Montana (Lavin, 2003).

1.2.2 Uses of *Populus*

Poplars have been used by humans since at least the dawn of civilization. People living in the Fertile Crescent more than 10,000 years ago used poplar wood for cooking and construction of dwellings (Stettler, 2009). The starchy sap found underneath the outer bark is edible to humans, and may have been used as a survival food during times of famine (Angier & Anderson, 1974). Today, poplars are used for timber, fiber, pulp, and environmental purposes such as windbreaks and erosion control (Isebrands & Richardson, 2013). Additional niche uses for poplars include the production of biomass for making third-generation biofuel and phytoremediation of contaminated soils (Harfouche et al., 2011).

Figure 1.2 A hybrid poplar plantation that was formally growing in Umatilla County, Oregon (*Hybrid Poplar Trees, Umatilla, Oregon*, 2005). Essentially all eastern Oregon plantations have been replaced by agricultural crops.

1.3 The genus *Eucalyptus*

1.3.1 Distribution and botanical description of *Eucalyptus*

The genus *Eucalyptus*, a member of the Myrtaceae family, contains over 700 species, all of which are native to the Australian continent or adjacent islands in Indonesia, Papua New Guinea, Timor-Leste, and the Philippines (CHAH, 2019). Eucalypts are distinguished from other genera of the myrtle family by their lack of petals and the presence of opercula that encapsulates the floral bud (Boland et al., 1984). *Eucalyptus* was first described in 1789 by French botanist Antoine Laurent de Jussieu using a specimen collected in Tasmania by James Cook during his third voyage to the Australian continent (Jussieu et al., 1789).

Typical eucalypt trees vary in height from 10 meters (33 ft) to 60 m (200 ft). *Eucalyptus regnans* is the tallest flowering plant on Earth, reaching 100 meters (330 ft) in height (*Australia's Champion Trees*, n.d.). Many *Eucalyptus* species lose their dead bark each year, revealing a new layer of green, white, or red living bark (EUCLID, n.d.). In some species, bark cells are able to photosynthesize in the absence of foliage (Eyles et al., 2009). Nearly all eucalypts are evergreen, but some species may lose their leaves at the end of a dry season. Leaves of mature eucalypts are

usually lanceolate, petiolate, and alternately arranged. Also, leaves have a glossy appearance due to their waxy cuticle (Boland et al., 1984).

Eucalypts are easily recognized by their distinctive flowers and fruit (called "gumnuts"). Stamens are encapsulated in an operculum, which is comprised of fused sepals, petals, or both (EUCLID, n.d.). Flowers lack petals but have many showy stamens. As the stamens expand, the operculum is forced off of the base of the flower. Eucalypt fruits are woody capsules shaped like a cone and have valves at one end to release the seeds. Seeds are small (~ 1 mm in length), waxy, and oblong (Boland et al., 1984).

Eucalypts were first brought from Australia to the rest of the world following Cook's third voyage to Australia in 1777 (L'Héritier, 1788). They have since been introduced to southern Europe, Africa, Western Asia, South Asia, South America, and California (Baldwin et al., 2012). Today, commercial *Eucalyptus* is the most widely planted tropical and sub-tropical hardwood tree in the world, covering roughly 20 million hectares in at least 90 countries worldwide. Brazil (5.7 m ha), China (4.5m ha), and India (3.9 m ha) comprise just over 70% of this area (CIRAD-FRA et al., 2018). The main commercially-grown species are *E. grandis* x *E. urophylla*, *E. urophylla*, *E. camaldulensis*, *E. tereticornis*, and *E. globulus*.

Figure 1.3 *Eucalyptus tereticornis* buds, capsules, flowers and foliage, Rockhampton, Queensland (Aardvark, 2008).

1.3.2 Uses of *Eucalyptus*

For millennia, aboriginal Australians have used the seeds of *Eucalyptus* as a food source and its oil as a topical antiseptic (Nangala et al, 2019; Palombo et al, 2001). The oil of *Eucalyptus* is prized in other parts of the world for its applications in food, fragrance, and medicine. In the developed world, it is commonly used as an ingredient in decongestants and perfumes (Clarke, 2008). Additionally, *Eucalyptus* plantations provide raw materials for a number of industries including pulp, charcoal, sawn timber, and wood panels. Due to its competitive advantage relative to other tree species in many areas with respect to biomass productivity, biotic and abiotic stress tolerance, and wood quality for short-fiber pulp, *Eucalyptus* plantations have expanded considerably in recent decades (CIRAD-FRA et al., 2018).

Figure 1.4 A eucalypt plantation in western Kenya (Shepherd, 2017).

1.4 Genetic modification in plants: Public acceptance and U.S. regulations

1.4.1 Brief history of genetic modification in plants

Even though natural selection was discovered less than two centuries ago (Darwin, 1859), humans have directed the evolution of many plants in ways that benefit the production of food, fiber, and psychotropic substances for millennia (Ren et al., 2021). This process was thought to begin when hunter-gatherers began sowing seeds of wild cereals like barley and einkorn in the early Holocene (c. 8500 BCE) in Southwest Asia. These early farmers would save the seeds of the best-performing plants for the next sowing, thus facilitating domestication through 'artificial selection' (Barker, 2006). This type of genetic improvement and the intentional sexual crossing of parental lines to create elite cultivars (termed 'breeding'), remain the primary methods of crop improvement today. Additionally, the advent of atomic energy in the 20th century brought about mutagenic breeding, which uses gamma-radiation to induce non-specific mutations in plant

genomes, a process which has yielded many useful varieties to date (Broad, 2007). However, both of these methods rely on random, indirect mutations as a source of genetic variance. Acquiring desired traits through these methods can be slow or impossible, especially if the genetic variation within a species is low (Govindaraj et al., 2015).

Just fifty years ago, Paul Berg's research group at Stanford proved experimentally that DNA could be cut from the genome of one species and pasted into that of another (Jackson et al., 1972). Later, they coined the term 'recombinant DNA' (rDNA) to describe the newly formed chimeric sequence. With this discovery, humans possessed the ability to make direct and specific asexual alterations in the DNA of living organisms (termed 'genetic engineering' or 'GE') (Williamson, 1952). Today, novel genes may be inserted into a plant genome, native genes may be removed or rendered dysfunctional, and even single-nucleotide edits can be made *in vivo* (Krens et al., 1982; Anzalone et al., 2020). This technology has made available many plant traits which would be impossible to acquire through sexual breeding or mutagenesis. The ability to introduce such a wide variety of novel traits, unconstrained by sexual compatibility, presents an unparalleled opportunity for genetic improvement in agriculture and forestry. It is my belief that this technology will help meet the demands of the climate emergency and a growing human population which is projected to surpass 10 billion in the next 30 years (UN, 2021).

1.4.2 The first 25 years: looking at the impact of GE crops

Since the first GE crop, the FLAVR SAVR™ tomato, was commercialized in 1994 (Kramer & Redenbaugh, 1994), the annual acreage of genetically modified crops has grown to at least 185 million hectares globally (Aldemita et al., 2015). These crops predominantly carry bacterial genes which confer resistance to herbicides and insect herbivory that have dramatically enhanced agronomic performance, leading to high adoption rates by farmers. It is estimated that more than 90% of the maize, cotton, soybeans, and sugar beets grown in the U.S. are GE, as well as >90% of soybeans in Brazil and oilseed rape in Canada (James, 2014). A meta-analysis of 147 agronomical studies of the early adoption of GE crops found that farmers' profits increased by 68% on average when using GE crops compared to conventional, and most of this increase came from developing countries. During this same period, global crop yields increased by 22%, and the annual cost of pesticides declined by 39% (Klümper & Qaim, 2014).

The widespread adoption of GE crops by farmers in the U.S. and elsewhere has led to significant reductions in pesticide use, carbon emissions, and has facilitated the switch to less toxic herbicides. From 1996 to 2018, total pesticide use in the U.S. decreased by 8.6% as a direct result of farmers choosing to plant insect-resistant GE corn, soybean, and cotton. This shift reduced the environmental impact of pesticide use (reported as the Environmental Impact Quotient (EIQ)) by 19%. During the same period, planting herbicide-tolerant GE crops shifted weed control tactics away from tillage, resulting in a carbon dioxide savings equivalent to removing 15 million cars from the roads. While herbicide use increased slightly during this period, the shift to less toxic herbicides like glyphosate reduced the EIQ associated with herbicide application by 12.9%. Perhaps most significant is that without the yield benefit from GE crops, an additional 14% of the arable land in the U.S. and 38% of arable land in Brazil would have been ploughed but has instead remained as ecologically-diverse grasslands and forestlands (Brookes & Barfoot, 2020).

1.4.3 Brief summary of U.S. regulation of GE plants

In the United States, GE plants are regulated by three regulatory agencies: the Environmental Protection Agency (EPA), the Food and Drug Administration (FDA), and the U.S. Department of Agriculture (USDA). Biotoxins, such as *Bt*, when they are used to increase pest resistance, are regulated by the EPA under their authority to regulate pesticides. The EPA requires developers to conduct a food- and environmental-safety analysis to ensure that the toxin is safe for humans and the environment, and that it won't pose an allergenic threat. The FDA operates the "Plant Biotechnology Consultation Program," a voluntary consultation process in which the developer (usually a company or university) consults with the FDA to ensure that products intended for human or animal consumption do not pose a human or animal health risk. The FDA does not conduct tests on the product, but relies on safety assessment data supplied by the developer (Food and Drug Administration, 2022). Since 1992, the FDA has considered most GE crops as "substantially equivalent" to non-GE crops and as such, crops do not require pre-market approval nor labeling. However, if a GE crop expresses a protein which differs significantly in structure or function from naturally-expressed proteins, and these are of nutritional or food safety consequence, the FDA may require pre-market approval and regulate

the protein as a novel food additive and require a label ("U.S. Regulation of Genetically Modified Crops," n.d.).

USDA regulations were substantially revised in 2020 for the first time in decades. The Sustainable, Ecological, Consistent, Uniform, Responsible, and Efficient (SECURE) rule was adopted, which 1) establishes exemptions for plants modified by genetic engineering techniques where the modification could otherwise be achieved through conventional breeding, 2) uses a risk-based approach to determine whether or not an organism is regulated instead of using a blanket "plant-pest trigger," and 3) provides a mechanism for a rapid initial review to distinguish plants developed using genetic engineering that do not pose plausible pathways to increased plant pest risk from those that do, and thus require further evaluation (Movement of Certain Genetically Engineered Organisms, 2020).

Under the SECURE rule, the regulatory trigger is no longer based on the use of plant pest vectors for genetic modification but is instead based on the use of the recombinant DNA method. However, final regulation depends on the product and its likelihood to be of plant pest concern. Additionally, simple gene-editing applications such as loss-of-function mutations in single genes using CRISPR/Cas9, will no longer be regulated, as they could be achieved using conventional breeding. However, more complex type of edits such as those using a template to guide mutations, or multiple loss-of-function mutations, will not be considered exempt. Another benefit of SECURE for developers is that regulatory decisions can be made for categories of modified crops under their Mode of Action provisions, not just for single gene insertions (Hoffman, 2021). SECURE was intended to lessen the regulatory burden of developing GE crops compared to the system it replaced, however, there is still a great deal of discretion USDA can apply to decisions, and thus the ultimate outcome remains unclear.

1.4.4 Market barriers for GE crops and trees

The National Bioengineered Food Disclosure Standard (NBFDS), which was passed by Congress in 2016, directed the USDA to establish a mandatory standard for disclosing foods which are or may be bioengineered (Agricultural Marketing Service, 2019). Per the standard, a bioengineered food "contains genetic material that has been modified through *in vitro* rDNA techniques and for which the modification could not otherwise be obtained through conventional breeding or found in nature." This standard went into effect at the national level on January 1st,

2022 (Hernandez, 2022). The NBFDS will be the first national standard of its kind to take effect in the U.S., but food labeling with regard to genetically modified organisms ('GMOs') is not new.

Before the NBFDS, the private sector had already established its own labeling programs in response to growing public concern over GMO foods. Perhaps the most notable example is the "Non-GMO Project," which had certified nearly 18,000 products from more than 1,200 brands as of 2014. In that same year, non-GMO labels were one of the fastest growing consumer trends within the food sector (Bain & Dandachi, 2014). Some believe that labeling some foods as non-GMO may make foods without the label appear more dangerous, but research has not yet substantiated that claim (Maghari & Ardekani, 2011).

Compared to GE food crops, forest tree biotechnology still faces immense market barriers. As of the writing of this thesis, the Forest Stewardship Council (FSC), a trans-national sustainable forestry certification entity, does not allow certification of companies which grow GE trees. This policy also extends to breeding activities. FSC does allow member firms to conduct limited field trials of GE trees, but those trees cannot be used in certified products (FSC, 2022). Because of the importance of FSC certification, this policy has kept almost all forestry companies from conducting field research on GE trees, even though many scientists believe GE trees could help address the increasing pressures that face plantation forestry, such as high incidences of insects, disease, and other pressures which have been brought about by a rapidly changing climate (Stokstad, 2019).

As of December, 2021, nearly 700 scientists and concerned citizens have signed a petition directed toward the FSC in the hope that the organization will revisit and overturn its GE research ban (Conrow, 2021). In response to the apparent petition, several anti-GE organizations, including the Global Justice Ecology Project (GJEP), sent a letter to the FSC urging the organization to maintain its ban. Additionally, the GJEP urged the FSC to eject member companies who wish to conduct GE tree research. GJEP officials cited possible environmental and social impacts of GE pollen spread as their reasons for opposing the policy change, emphasizing that "[b]ecause of the complex nature of trees and forests, [the] risks are impossible to thoroughly assess" (Global Justice Ecology Project, 2021).

1.4.5 Factors that influence public attitudes toward GE plants

While farmers in the US and elsewhere have readily adopted GE crops, consumers remain less enthusiastic. Almost immediately after being placed on the market, Greenpeace and other non-governmental organizations (NGOs) began campaigning against the use of GE crops (Paarlberg, 2014). Citing a lack of scientific evidence to prove their safety, some NGOs go as far as saying GE crops should be banned (Paarlberg, 2014; Slow Food, n.d.). Since the release of GE crops, 4,400 separate risk assessments carried out in over 70 countries have unanimously found no increased risk of consuming GE crops versus conventionally-bred crops (ISAAA, 2019). Likewise, an estimated 70-90% of these GE crops go to animal feed, and since 1996, no adverse effects have been recorded in the more than 100 billion animals fed using predominantly GE feed (Van Eenennaam & Young, 2014). An important consideration in public decision-making is that NGOs such as Greenpeace are generally more trusted by the public than institutions such as biotechnology firms and the government entities responsible for regulating GE crops (Bernauer & Meins, 2003; Bonny, 2003; Hilton & Mouhot, 2013; Vilella-Vila & Costa-Font, 2008; Zilberman et al., 2013). Furthermore, the public is not well-acquainted with the scientific process (Miller, 1998), necessitating public reliance on interpretations of scientific data by people and organizations that they trust. This may lead to persuasion by ideological actors (e.g., NGOs, ideological actors, etc.), which may contribute to what I call the 'Fernbach phenomenon.' In a random sampling of US adults, Fernbach et al. found that "as extremity of opposition to and concern about genetically modified foods increases, objective knowledge about science and genetics decreases, but perceived understanding of genetically [engineered] foods increases" (Fernbach et al., 2019). Since governments and certifying organizations are likely to be influenced by strong negative attitudes of the public toward GE crops when crafting regulations, it is important that the public be well informed of the true personal and societal risks of GE crops (European Commission, n.d.; Wolt & Wolf, 2018).

There are several factors that may lead people to be cautious about GE food and products. For one, the reputation of GE may be forever entwined with the reputation of the companies that produced them. Emery (2020) found that people "rely on their knowledge and beliefs about agricultural biotechnology to inform their beliefs and attitudes toward forest biotechnology." Perhaps most prominently, the Monsanto Company (now Bayer AG) already suffered from poor

public perception prior to the release of GE seed as a consequence of prior controversies involving several chemical products such as the herbicide ‘Agent Orange’ (Rebière & Mavoori, 2020). Secondly, there seems to be common apprehension about the idea of consuming ‘foreign’ DNA originating from bacteria or plant viruses. However, since humans consume ‘foreign’ DNA each time we eat (due to native plant microbes in food), this concern is mostly without scientific merit. Further, DNA of all origins (including from our own cells) is readily degraded during digestion (Jonas et al., 2001). Another proposed concern with consuming GE food is the presence of enzymes which confer antibiotic resistance, a relic of genetic transformation, which some feared would lead to antibiotic-resistant gut microbes. However, experiments found that the bacterial nptII enzyme, the most commonly used antibiotic resistance marker, is rapidly degraded in mammalian guts before it reaches the intestine, even at million-fold levels of what a person would consume eating GE potatoes which produce the nptII enzyme (Fuchs et al., 1993).

In addition to these claims, multiple other consumer safety concerns have been dismissed for lack of evidence, including horizontal gene transfer to microbes (Hull et al., 2000), reduced nutritional quality (Duke et al., 2003; El Sanhoty et al., 2004; Li et al., 2007; Ridley et al., 2002), and allergenicity (rev. in Batista & Oliveira, 2009). Not to be dismissed, however, is the concern of allergenicity. In 1996, it was found that a protein from Brazil nut which was added to GE soybeans would cause allergic reactions in a subset of consumers (Nordlee et al., 1996), but the GE soybeans harboring this protein were subsequently never marketed. Additionally, a novel insecticidal protein, Cry9c, was implicated in causing allergic reactions in people who ate GE StarLink maize, although the evidence for this remains unclear (Sutton et al., 2003). The primary reason that GE products are not often implicated in promoting allergic reactions is the rigorous testing and bioinformatic analyses undertaken by developers for FDA or EPA review prior to approving novel GE products for human consumption (König et al., 2004). Developers look at the amino acid sequence of the novel protein product and compare it to that of known toxins and allergens, conduct acute toxicology studies with rodents, and assess digestibility in conditions which mimic the human stomach (Quemada et al., 2010).

The addition of lepidopteran-targeting insecticidal proteins from *Bacillus thuringiensis (Bt)* to many GE crops has generated a lot of hesitancy by the public (Stone, 2011). Hesitancy surrounding *Bt* is two-pronged: people fear the addition of an insecticidal protein to food which

they will consume, and they fear that the protein will harm 'off-target' lepidopteran species (e.g., the monarch butterfly). While *Bt* is toxic to the larvae of lepidopterans (moths and butterflies), their stomach conditions are entirely different from mammalian stomachs (Stumpp et al., 2015). In fact, the *Bt* protein is fully digested within 30 seconds in conditions mimicking the human stomach (Quemada et al., 2010). Addressing the concern about 'off-target' harms, initial reports implied that corn pollen which contains *Bt* might be responsible for the decline of the monarch butterfly (Losey et al., 1999). However, follow-up studies found that the *Bt* gene is not expressed highly enough in pollen to constitute a significant threat (Sears et al., 2001).

Attitudes towards GE food crops are not limited to concerns over human health. As with forest biotechnology, environmental health is also a major concern of many consumers and even farmers. Of particular concern to farmers is the increase in glyphosate-resistant weeds. Glyphosate-resistant (GR) crops were first marketed in the 1990s, and glyphosate use has increased as glyphosate-resistant crops now dominate the American market (James, 2014). One reason GR crops were so readily adopted by farmers in the 1990s was existing herbicide-tolerance to many other herbicides in weeds. Glyphosate, a low-cost, broad-spectrum herbicide, gave good control of these weeds at the time. However, repeated exposure to glyphosate which was sprayed 'over-top' of GR crops led to the demise of its efficacy as natural mutations in weed species coupled with natural selection led to resistance (Bonny, 2016). In 2017, there were 17 species of glyphosate-resistant weeds in the US, and many more reported globally (Heap & Duke, 2018). This apparent failure is not due to the technology, but rather to the irresponsible overreliance on glyphosate by farmers over the course of decades.

Few studies have looked at public attitudes towards GE forest trees specifically, although public opinion seems to depend on the context in which GE trees are used. Among a sample of mostly European students, the use of GE trees in plantations was much more favored than in wild forests (Kazana et al., 2016). In a sample of U.S. adults, it was found that people were less likely to support the use of GE trees in wild forests than conventional techniques (e.g., breeding, planting, thinning), and inserting native genes ('cis-genesis') was more acceptable than exotic genes from another species. Additionally, there was more favorability for using GE to address diseases than other issues such as increasing growth rate or helping address climate impacts (Needham et at., 2015). A questionnaire study by Petit et al., which included a random

representative group of U.S. adults and a forest interest group (e.g., forest scientists, managers), found that both groups had positive attitudes toward using GE to restore the American chestnut, but the expert group had more favorable attitudes than the randomly sampled group. Perceived risks and benefits were the largest drivers of attitude for both groups, and the expert group perceived greater benefits and lower risks than the random sample group (Petit et al., 2021). This finding is consistent with previous research which also found more favorable attitudes toward GE than the public at large (Małyska et al., 2014; Porth & El-Kassaby, 2014).

A series of qualitative discussions with individuals in the Pacific Northwest region suggests that the public is most concerned about potential "unintended ecological consequences that might arise from forest biotechnology" including invasiveness and gene flow (Emery, 2020). Unlike most agricultural crops, forest trees are capable of dispersing pollen many miles away, which creates a high relative risk for gene flow from GE trees to wild trees (Strauss et al., 2017). Also unlike GE agricultural crops, forest trees are often planted near their center of origin, and so a high amount of gene flow would be expected from fertile GE trees to wild relatives. However, these concerns do not appear to take into account the considerable progress made toward genetic containment by sterility in forest trees, which would eliminate the spread of GE traits to wild relatives (Elorriaga et al., 2021; Goralogia et al., 2021; Klocko et al., 2021). It remains to be seen how so-called 'engineered sterility' will impact public perception of GE forest trees (Strauss et al., 2017).

Public concern over GE crops and forest trees appears to originate from a high perceived risk to perceived benefit ratio, which is the foundation of rational decision making in humans and animals (Reyna, 2004). However, the public has often overestimated the risk posed by GE technology, perhaps due in-part to ideological actors like Greenpeace and other NGOs. The vast majority of scientific studies on the safety of GE food and products have found no increased risk versus their conventionally-bred counterparts. However, that does not mean that there are not risks associated with this technology, as discussed. Science-based regulations and risk/benefit analysis must continue to ensure that neither humanity nor environment are harmed by this relatively novel and powerful technology.

1.5 Genetic improvement in trees

1.5.1 Conventional tree breeding

Unlike most other crops plants such as cereals, fruits, and vegetables, for the majority of forest tree species domestication and systematic breeding has only recently begun. Forestry breeding formally began in the U.S. and Europe in the 1950s following high demand for structural wood and diminished natural stands (Lebedev et al., 2020; Paques, 2013).

Due to their recently begun domestication, forest trees benefit from broad genetic variability owing in large part to the wide geographic ranges of many species. Beginning in the 1940s, the study of this genetic variability began with sampling of forest tree populations from across their natural range followed by planting test sites (also known as 'provenance trials'). This took place across Europe and the U.S. Provenance trails are used to evaluate which trees are suitable for a specific locale, as widespread species tend to be locally adapted. This local adaptation is also known as 'adaptive divergence' (Clausen, 1940). These early provenance trials focused mainly on important conifer species such as Eurasian larch species, pines, and Douglas fir. However, provenance trials continue to comprise the first step in many tree domestication and breeding programs today, including *Eucalyptus* (Carnegie & Keane, 2003), *Populus* (Uniyal & Todaria, 2006), Sakhalin fir (Ishizuka et al., 2021), and others (Risk et al., 2021). Provenance trials often define the 'base populations' in a breeding program, from which breeders will assess phenotypic traits and make selections for the following steps in the breeding cycle (Wheeler & David, 2011). Transfers between distinct provenances are rare, though climate change pressures are prompting many programs to now consider much wider movement, often termed "assisted migration" (Aitken & Whitlock, 2013).

The breeding cycle

The breeding cycle is based on recurrent selection, which is a cycle of selection followed by intermating of selected individuals over multiple generations (Lidder & Sonnino, 2012). The breeding cycle consists of four primary stages (from White et al., 2007):

1) Selection – where a subset of the base population (or provenance trial) is selected for breeding by a breeder. At this stage, breeders must make decisions regarding relative

emphasis to be placed on a wide variety of phenotypic traits. The goal of selection is to increase the frequency of desirable alleles in the breeding population.

2) Breeding – the mating of trees with desirable phenotypic traits results in recombined genotypes which may contain a novel portfolio of traits. The tree breeder's broad goal is to increase the frequency of favorable alleles (a concept known as 'genetic gain') while retaining or enhancing genetic diversity at all other loci (Wheeler & David, 2011). It should be noted that tree breeding requires careful pedigree management to avoid inbreeding depression, a phenomenon which can result in reduced vigor and fertility (Williams, 1996).

3) Testing – Following breeding, field trials of progeny are tested for performance under the intended climatic conditions in order to measure genotype x environment (G x E) interactions, genetic gain, and other metrics. At this stage, breeders make further parental selections for the next breeding cycle. This stage concludes the three-part process of recurrent selection.

4) Production – If enough genetic gain has been captured by the breeding process, cuttings of proven lines which are known to produce good-quality offspring are planted in clonal seed orchards (aka 'clone banks') to expand their numbers. Progeny seedlings from these trees will then be deployed in the growing area. For production of *Populus*, *Eucalyptus*, and *Acacia*, cuttings are taken directly from the proven lines and planted in large-scale plantations (Dickmann et al., 2001; Doughty, 2000; Koutika & Richardson, 2019)—which allows better use of non-additive genetic variance and more rapid genetic gains (Libby & Rauter, 1984).

<u>New technologies may speed up genetic gain</u>

The major constraint to tree improvement is the significant length of the breeding cycle due to delayed onset of reproduction, which can be 20-30 years for some species (Lebedev et al., 2020)—though is usually closer to 5-10 years under high light and fertility conditions. Much of this time is spent visually evaluating phenotypes, which takes a similar period of time for reliable assessments (usually at least 5 years). New genomic tools such as genomic selection (GS),and marker-assisted selection (MAS) that allow for genotypic rather than phenotypic selection have

been widely deployed in crop breeding and GS has begun to be used in forest tree breeding (Boopathi, 2020; Grattapaglia, 2017; Grattapaglia & Resende, 2011; Muranty et al., 2014).

MAS selects trees based on one or a few genes, where GS selects trees based on a sample of the entire genome. MAS has found limited application in forest tree breeding in part because MAS functions by exploiting linkage disequilibrium between marker and quantitative trait loci (QTL), which works best for simple traits (e.g., disease resistance) controlled by only a few QTLs (El-Kassaby, 1982). Unfortunately, the most economically significant traits for forest trees (such as height, wood quality, and canopy architecture) have complex and quantitative inheritance patterns. For these traits, MAS is not very useful (Lebedev et al., 2020).

GS analyzes all gene effects regardless of significance (Meuwissen et al., 2001). The main difference between MAS and GS is that GS uses vastly more markers that cover all chromosomes (often tens of thousands of genes). For forest trees, GS will have much higher prediction accuracy than MAS as so few major-effect QTL have been identified (Thistlethwaite et al., 2019). The main advantage of GS for phenotypic selection is the ability to shorten the selection period of the breeding cycle based on a training model developed in a prior generation.

Marker-assisted assessment of genotypes (be it MAS or GS) can be completed virtually as soon as DNA can be extracted and sequenced without harming the plant. Compared to long and costly field trials of progeny, genomic selection allows breeders to quickly and cheaply evaluate traits that may take decades to fully manifest in the field. Assuming there is also a means for accelerating flowering such as top grafting, this could lead to massive improvement in genetic gain over time simply by reducing the time of each breeding cycle (Bhat et al., 2016). For eucalyptus, GS has been demonstrated to cut the breeding cycle in half (Grattapaglia, 2017). However, use of GS in conifers is fairly recent due to the redundancy and size of most conifer genomes (Howe et al., 2020).

1.5.2 Use of plant tissue culture in forestry

Plant tissue culture (PTC) is broadly defined as maintaining or multiplying plant tissues (protoplast, plant cell, tissue explants, plant organs) in a sterile, nutrient-rich medium within an aseptic environment, commonly referred to as *in vitro* tissue culture (K. S. Kumar & Ponmurugan, 2012). German botanist Gottlieb Haberlandt is credited with the discovery of

aseptic plant tissue culture. Using a simple medium containing glucose, peptone, and knop's salt, Haberlandt was able to culture isolated plant cells *in vitro* (Haberlandt, 1904).

Plant tissue cultures are initiated from small pieces of plant tissue, called explants, which may be taken from practically any part of a plant. Exogenous hormones, notably synthetic auxins and cytokinins, are added in order to induce the formation of undifferentiated cell clusters called callus. Callus cells must then be coaxed into forming meristematic buds that develop into a shoot meristem, which is a small group of cells that give rise to new organs (Ha et al., 2010). This process, called 'organogenesis,' was first accomplished in forest trees more than 50 years ago in *Populus* tissue culture, marking the beginning of *in vitro* propagation of forest trees (Winton, 1968). Shoot meristems are then placed in rooting medium before their eventual transplantation to the field. Called 'micropropagation,' many thousands or millions of clonal plantlets may be regenerated from a single explant in this fashion (Bhojwani & Dantu, 2013; Murashige, 1974).

The application of micropropagation in clonal forestry is immediately evident and holds promise for forest tree breeding (Bonga & Durzan, 1987; López-Peralta & Sánchez-Cabrera, 1991). As previously mentioned, clone banks are used to multiply single genotypes for both breeding purposes and pre-deployment production. This is especially true for species which are planted in genotypically-uniform plantations such as *Populus*, *Eucalyptus*, and *Acacia*. However, this requires many acres and older trees may not produce easily-propagated cuttings (Ahuja, 1993). Contrastingly, micropropagation can produce many millions of elite clonal plants each year from only a small amount of plant tissue, saving precious time in the final production phase of the breeding cycle. Additionally, micropropagation of plantlets is not dependent on season or daylength, so it can even be accomplished in the winter.

Other benefits of micropropagation are:

(a) Allows for multiplication of disease-free plants
(b) Allows multiplication of sexually-derived sterile hybrids (Idol et al., 2019)
(c) Cost effective and requires very little growing space

Somatic embryogenesis

While micropropagation works well for many agricultural crops and angiosperm forest trees, many conifers remain difficult to micropropagate (S. M. Jain & Häggman, 2007). An

alternative multiplication strategy is a process known as somatic embryogenesis, which involves the formation of embryogenic callus from somatic tissue (often called 'embryonic masses'). The embryonic masses then are allowed to proliferate before the embryos are allowed to mature into entire plantlets (Montalban et al., 2016). Somatic embryogenesis has been widely deployed in the commercial propagation of elite conifer clones (Lelu-Walter et al., 2013). Embryogenic cultures are simple yet time-consuming to maintain once established, but somatic embryogenesis is not without limitations. One of the primary drawbacks is the risk of contaminating the embryo culture during subculturing to fresh medium, necessitating that somatic embryogenesis be coupled with a cryopreservation protocol of the embryo culture (Lambardi & Ozudogru, 2008).

Somaclonal variation

Long-term culturing of plant cells in the presence of auxins and cytokinins can lead to gene and chromosome-level variations known as 'somaclonal variation.' Although it is a well-studied and widely-documented phenomenon, the causes of somaclonal variation remain undetermined (Bairu et al., 2011). It is well documented that long-term callus cultures derived from differentiated tissues such as leaf or stem typically give rise to more variation than meristematic cultures (Sharma et al., 2007).

While most somaclonal variation is seen as deleterious, it can sometimes produce useful mutations that improve the agronomic performance of a clone, such as enhanced disease resistance, abiotic stress response, yield, and others (M. S. Jain, 2001). In elite clonal varieties with deteriorating disease resistance such as the 'Russet Burbank' potato or the 'Cavendish' banana, somaclonal variation arising from tissue culture may constitute a significant source of much-needed genetic variation (Emaldi et al., 2004; Tegg et al., 2013). Due to the rather large genetic resources most forest tree species have, somaclonal variation is not used to introduce novel mutations and is generally seen as a negative consequence of tissue culture (Rani & Raina, 2000).

Tissue culture in genetic engineering applications

Following the insertion of exogenous DNA (transformation; detailed in the next section), the 'transformed' cells must undergo embryogenesis or organogenesis similarly to micropropagated tissues. Thus, tissue culture techniques are prerequisite to the majority of plant

genetic engineering methods, though there are exceptions, including for some trees (Maher et al., 2020; Zhang et al., 2017). Tissue culture in genetic engineering differs slightly, as selective agents such as antibiotics or herbicides must be added to arrest the development of non-transformed cells that could compete with transgenic cells for resources (Bhojwani & Dantu, 2013).

1.5.3 Genetic engineering in forestry

Increased demand for forest products and accompanying deforestation are major threats to biodiversity and the many social and environmental services that natural forests provide (Willis, 2003). Genetically improving forest trees and increasing plantation forestry are two major ways to reduce the strain on natural forests while meeting consumer demands (Walter, 2004). The transfer of genes with desirable phenotypes to elite cultivars by traditional breeding and recurrent selection can take decades in forest trees. However, genetic engineering (GE) can accomplish the same task in a single generation without the risk of 'carrying over' undesirable linked alleles in the process (Ahuja, 2011). Additionally, GE is not bound by sexual compatibility barriers, and genes from every kingdom, or those designed by humans based on scientific principles, may be introduced to forest trees.

Genetic engineering in forest trees began in the 1980s following breakthroughs in molecular biology that allowed the transformation of poplar using *Agrobacterium tumafaciens* (Fillatti et al., 1987). Since then, a host of forest tree species have been genetically engineered with modifications to growth traits, wood quality, disease and insect resistance, herbicide tolerance, sterility, and more (reviewed in Harfouche et al., 2011). However, despite many field trials of various forms of insect-resistant trees in China, only one genotype, a Bt-pest-resistant poplar, has been "commercialized" as of 2022 (Ewald et al., 2006), and appears to be cultivated only on a very limited scale (Wang et al., 2018). More GE trees were expected to be commercialized or released in the U.S., including a frost-tolerant *Eucalyptus* hybrid, a blight-resistant American chestnut (*Castanea dentata*) and a loblolly pine (*Pinus taeda*) with increased wood density (Carlson et al., 2022; Z. D. Miller et al., 2019; Wear et al., 2015). However, only the chestnut tree is being actively pursued at present due to a variety of economic and regulatory obstacles.

Global climate change will likely shift the suitable ranges of forest trees and place stress on many current plantations (Davis et al., 2005; Iverson et al., 2019). Due to the slow rate of breeding in most species, it is conceivable that GE is a uniquely promising tool to address this problem. Introducing drought-tolerance genes from better-adapted plants to forest trees might be one way mitigate the effects of future droughts. Drought-tolerance is a complex trait with no easy GE solutions (Nuccio et al., 2018), though much progress has been made identifying target genes such as the chickpea *CBF1* transcription factor (Liu et al., 2017) and *hahb-4* ("Argentina First to Market with Drought-Resistant GM Wheat," 2021). As temperatures change, insect pest pressure may increase as well (Skendžić et al., 2021), which is something GE is well-equipped to deal with (Bakhsh et al., 2015). Climate change will bring other challenges to forestry as well, and an excellent review has been written about how GE might play a role in negating some of its effects (Ahuja, 2021).

GE may be able to introduce novel traits to a breeding program, but it is not a substitute for traditional breeding. In reality, only a handful of genotypes are readily transformed, necessitating backcrossing a transformed genotype with GE traits to elite cultivars as part of the breeding cycle. Thus, GE of forest trees is still limited by the speed of the breeding cycle. Of course, this limitation could be overcome if there is progress made in developing transformation systems that work in elite cultivars or genotype-independent transformation systems.

1.6 *In vitro* plant regeneration mechanisms

Regeneration of tissues or organs following bodily injury is a widely conserved mechanism among plants and animals (Pulianmackal et al., 2014). Plants possess an extraordinary capacity for regeneration, which has enabled clonal propagation and grafting (Hartmann et al., 2002; Melnyk et al., 2015). Following the invention of plant tissue culture at the beginning of the 20th Century by Haberlandt (1902), a breakthrough discovery was made that exogenously applied plant hormones, auxin and cytokinin, largely determine the fate of regenerating tissues. A high auxin to cytokinin ration promoted root regeneration, while a high cytokinin to auxin ratio promoted shoot regeneration (Skoog & Miller, 1957). This discovery was prerequisite to plant biotechnology, which requires the regeneration of newly-transformed plants. In this section, the cellular mechanisms of regeneration will be discussed.

1.6.1 Plant regeneration through *de novo* shoot organogenesis

Plants may be regenerated *in vitro* by the formation of new meristems, which are regions of cells responsible for new growth in plants (Encyclopaedia Britannica, 2020). Plants have three types of meristems: root, shoot, and floral, which give rise to the three main organ types (Hüner & Hopkins, 2009). The process of regenerating new shoot meristems is termed *de novo* shoot organogenesis (DNSO) from the latin phase which means 'anew' (Merriam-Webster, n.d.).

DNSO is a two-phased process. In the first phase, so-called 'pluripotent' cells must be produced. To induce cell pluripotency, plant tissues are cultured on a high-auxin callus induction medium (CIM). Callus cells arise from the division of xylem pole pericycle cells in a process which strongly resembles the formation of lateral root meristems (LRM) (Atta et al., 2009; Sugimoto et al., 2019). After callus tissue is formed, a subpopulation of cells establishes a root stem cell niche (SCN), which expresses key regulators of root stem identity. Root meristem genes such as *WUSCHEL-RELATED HOMEOBOX 5 (WOX5), SCARECROW (SCR), PLETHORA 1 (PLT1), PLT2, ROOT CLAVATA-HOMOLOG 1 (RCH1),* and *SHORT ROOT (SHR),* are highly up-regulated in callus cells (Atta et al., 2009; Sugimoto et al., 2010). The expression of these genes is a key component of cell pluripotency acquisition, as exemplified by low DNSO rates in their mutant lines (J.-Y. Kim et al., 2018). Of these genes, *PLT1 & 2* seem especially crucial stem cell niche maintenance (Kornet & Scheres, 2009; Vlachonasios et al., 2003), while *WOX5* is vital for maintaining stem cell identity (Sarkar et al., 2007).

Following the acquisition of competence, callus cells must undergo several transformative processes before a shoot may be formed. The first process, in which a dome-shaped shoot 'promeristem' is formed, begins with high expression of the *CUP-SHAPED COTYLEDON 1* (*CUC1*) and *CUC2*. *CUC1/2* are upregulated by *ENHANCER OF SHOOT REGENERATION 1* (*ESR1*), which accumulates rapidly after competency acquisition on CIM (Ikeda et al., 2006; Matsuo et al., 2011). Once explants are placed on shoot-induction medium (SIM), *PLT3, PLT5,* and *PLT7* further upregulate *CUC1/2* (Kareem et al., 2015), which in turn promotes the expression of *SHOOT MERISTEMLESS* (*STM*), a master-regulator of shoot meristem maintenance (Daimon et al., 2003).

After a promeristem is formed, the next process is the formation of a shoot meristem primordium, the progenitor of a shoot meristem. The conversion of a shoot promeristem to a

primordium begins with the activation of *WUSCHEL* (*WUS*) expression by cytokinin upon transfer to SIM (Gordon et al., 2007). *WUS* is a master-regulator of shoot meristem identity, and represses the auxin-induced expression of root meristem cells in the shoot promeristem to promote shoot regeneration (Negin et al., 2017). *WUS* expression is regulated by epigenetic modifications during DNSO (Ishihara et al., 2019; Li et al., 2011) and *ARABIDOPSIS RESPONSE REGULATOR* (*ARR*) *1,2,10*, and *12*, which bind directly to the *WUS* promoter and activate its expression on SIM (T.-Q. Zhang et al., 2017). Other intricate signaling interactions involved in *WUS* expression are beyond the scope of this review, but are reviewed elsewhere (Shin et al., 2020; Sugimoto et al., 2019).

Once a shoot meristem primordium formation is complete, the shoot can begin growing and developing. Expression of leaf identity genes is governed by miRNA165 and miRNA166, which target the *HD-ZIP III* family genes, including *PHABULOSA* (*PHB*), *PHAVOLUTA* (*PHV*), *REVOLUTA* (*REV*), *KANADI* (*KAN*), and *ARABIDOPSIS THALIANA HOMEOBOX GENE 8* (*ATHB8*) (Jung & Park, 2007; T. Liu et al., 2012; Z. Zhang & Zhang, 2012). Another player in leaf identity is the antagonistic interaction of Trithorax Group proteins and Polycomb Group proteins. The *Polycomb Repressive Complex 2* (*PRC2*), is down regulated during the callus-to-leaf transition during shoot regeneration (He et al., 2012; Lee et al., 2019). Other shoot developmental genes, including *SAWTOOTH 1* (*SAW1*), *SAW2*, and *TCP FAMILY TRANSCRIPTION FACTOR 10* (*TCP10*), are activated during late-stage DNSO (Lee et al., 2019).

1.6.2 Plant regeneration through somatic embryogenesis

Somatic embryogenesis (SE) is a process whereby a somatic plant cell can dedifferentiate to a totipotent embryonic stem cell that has the ability to give rise to an embryo under appropriate conditions (Guan et al., 2016). SE can be induced by a wide range of abiotic stressors *in vitro* (Fehér, 2015) and by exposure to an auxin-rich medium (Wernicke & Brettell, 1980). There are many types of synthetic auxin, but 2,4-dichlorophenoxyacetic acid (2,4-D) is the most effective at inducing SE, perhaps due to its ability to stimulate both the auxin-response and the stress-response pathways simultaneously (Gliwicka et al., 2013).

During indirect SE, the most common method for SE formation, explant tissues are first placed in an auxin-rich medium to promote the proliferation of embryonic callus (Ikeda-Iwai,

2002). Then, explants are transferred to an auxin-free medium, where an auxin-gradient is established in the embryonic callus. This gradient initiates a cellular program which closely mimics zygotic embryogenesis (Cm. Liu et al., 1993; Su et al., 2009), and culminates in the localization of *WUS* expression in the low-auxin domains which will later become the shoot meristem (Su et al., 2009).

Other master regulators of SE include *LEAFY-COTYLEDON 1* (*LEC1*) and *LEC2*. *LEC1/2* are involved in inducing expression of the *YUCCA* family of auxin biosynthesis enzymes (S. Braybrook & Harada, 2008; Junker et al., 2012). Additionally, *LEC2* promotes *INDOLE ACETIC ACID INDUCIBLE30* expression, which modulates auxin-mediated signaling (Braybrook et al., 2006). A dramatic example of the master-regulatory role of *LEC1* in SE comes from the genus *Kalanchoë*, known as the "mother of thousands." Several members of the genus produce entire SE-derived plantlets on their leaf margins, owed entirely to a defective natural *LEC1* gene (Garcês et al., 2007).

1.7 Overcoming transformation and regeneration bottlenecks in food crops and forest trees using developmental regulatory ('DEV') genes

Genetic engineering of plants lagged behind that of bacteria (Jackson et al., 1972), animals (Jaenisch & Mintz, 1974), and yeast (Hinnen et al., 1978). This is in part because, unlike the protein-rich extracellular matrix of bacterial and animal cells, plant cells are encapsulated in a rigid polysaccharide cell wall (Baker, 1952) which presents a physical barrier that complicates the insertion of exogenous DNA. The first experiments which successfully integrated new DNA into a plant genome (termed "stable" transformation) overcame this barrier by removing the cell wall through enzymatic digestion, yielding plant "protoplasts," a term derived from an Ancient Greek word which means "first-formed" (Hanstein, 1880). The DNA was delivered to protoplast cells by disarmed *Agrobacterium tumafaciens*, a bacterial plant parasite which has an evolved mechanism to insert tumor-inducing (Ti) genes into plant genomes (Krens et al., 1982). However, protoplasts are delicate and difficult to maintain in culture for long periods of time. A more general approach which works in a broader range of plant species involves co-cultivation of disarmed *A. tumafaciens* with differentiated explant tissues (Horsch, 1985). While there are alternative methods including particle bombardment (Klein et al., 1987), magnetic nanoparticle delivery (Zhao et al., 2017), and others (rev. in Lv et al., 2020), *Agrobacterium*-mediated

transformation (AMT) remains the simplest and most common method for introducing novel genetic material to plants, including gain-of-function trait genes and genome-editing machinery such as the much heralded CRISPR-Cas module (Jinek et al., 2012).

However, AMT remains challenging for many plant species, including conifers (S. Liu et al., 2020), fruit trees (G. Song et al., 2019), and many other species which do not benefit from well-established AMT protocols such as hops (Horakova et al., 2021).

Regeneration bottlenecks

The primary bottleneck for most crop species is no longer the stable integration of transgenes, but regenerating transgenic or gene-edited plants from transformed tissues (Altpeter et al., 2016). Regeneration is typically achieved by one of two methods: somatic embryogenesis or organogenesis (*de novo* meristem formation). Organogenesis is often preferable to embryogenesis due to both the technical difficulty of maintaining embryogenic cultures and somoclonal variation, which can result from the high levels2,4-D that are often required in embryogenic plant medium (Sala & Labra, 2003). Organogenesis involves placing the transformed explants on medium containing a specific auxin/cytokinin ratio which encourages *de novo* meristem formation (Ikeuchi et al., 2016; Murashige, 1974; Skoog & Miller, 1957). The exact auxin/cytokinin ratio which optimally promotes *de novo* meristem formation depends on species, genotype, and chemical form of the hormone (Raspor et al., 2021) and precise timing of hormone delivery (Smýkalová et al., 2019).

1.7.1 Transformation and regeneration bottlenecks in *Eucalyptus* and *Populus*

Challenges in *Eucalyptus* transformation and regeneration

Although genetic transformation of elite eucalypt genotypes has produced healthy trees with stable expression of valuable transgenes, the process of regenerating transformed cells in vitro is generally slow (at least 3-4 months), occurs at very low rates (<<10%), and is nearly impossible for some genotypes. Regeneration protocols must be customized for each genotype, which further increases the cost and difficulty of transformation (Chauhan et al., 2014). One of the most pernicious problems with obtaining transgenic shoots from eucalypts is the high degree of explant necrosis and death resulting from their release of phenolic compounds into the medium (Laine & David, 1994). Additionally, there is not a regenerable public genotype,

restricting public sector research on transgenic eucalypts. In summary, a more efficient, genotype-independent regeneration protocol is sought for *Eucalyptus.*

Challenges in *Populus* transformation and regeneration

Poplar is generally considered a model for biotechnology research in angiosperm trees. One reason for this may be the abundance of efficient poplar transformation protocols for a wide range of explant types. However, these protocols are limited to a small number of species and genotypes, many of which are not of commercial importance (rev. in Song et al., 2019). While some aspen genotypes tend to regenerate transgenic shoots at high rates (20-50%), transformation of cottonwood species is comparatively much less common and more challenging (K. H. Han et al., 1997; K.-H. Han et al., 2000). Many cottonwood genotypes are nearly completely recalcitrant, including the *Populus trichocarpa* genotype 'Nisqually-1,' the genotype which was used to construct the poplar genome (Tuskan et al., 2006). Similarl to eucalypts, regeneration protocols must be individually tailored for nearly every poplar genotype. A method which improves the transformability and regenerability of cottonwoods and other recalcitrant poplars would help advance transgenic research in the genus.

1.7.2 Overview of DEV genes commonly used in plant transformation

Difficulty in transformation and regeneration is not unique to *Eucalyptus* and *Populus,* or to forest trees generally. Many economically-significant plant species and genotypes remain difficult or impossible to transform and/or regenerate. One way to overcome this recalcitrance to regeneration is to overexpress genes which are involved in the transcriptional control of plant growth and development (Gordon-Kamm et al., 2019). The growth and development of plants is tightly controlled by a network of genes participating in feedback loops and signal cascades which respond to environmental and hormonal cues (Nagle et al., 2018). During *in vitro* regeneration, cells undergo reprograming which involves many developmental and cell identity genes (Ikeuchi et al., 2018). It is therefore logical to assume that overexpression of genes which promote meristem or embryo cell fates and/or cellular reprograming (termed "DEV" genes) will promote plant regeneration. Though this review will focus primarily on genes which have demonstrated usefulness or potential for usefulness in dicot plants, some monocots studies will be discussed.

The first such "DEV" gene was identified by Bryan and Sass in maize. In an ear-row planting of the open-pollinated variety Walden Dent, leaf blades produced ectopic shoot meristems which they termed "knots" (Bryan & Sass, 1941). The gene responsible for this phenotype was later discovered to be a homeodomain transcription factor, *Knotted-1* (Hake et al., 1989). When constitutively expressed in *Arabidopsis* or tobacco, the maize *Knotted-1* gene led to a proliferation of ectopic shoot meristems (Lincoln et al., 1994; Sinha et al., 1993). Later, it was discovered that the *Arabidopsis* ortholog of *Knotted-1, SHOOT MERISTEMLESS* (STM), also plays a crucial role in meristem formation and maintenance along with *CUP-SHAPED COTYLEDON 1* (CUC1), which is involved in a feedback loop with STM (Long et al., 1996; Takada et al., 2001). Furthermore, overexpression of CUC1 in *Arabidopsis* similarly led to a proliferation of ectopic shoot meristems (Daimon et al., 2003).

Of particular interest for use as a DEV gene, due to its central role in meristem formation and maintenance, is the *WUSCHEL* (WUS) family of homeodomain transcription factors. It was first discovered through loss-of-function mutants which strongly resembled *stm* mutants (Laux et al., 1996). WUS genes are expressed in the organizing center (OC) of the shoot apical meristem (SAM) before relocating to the central zone (CZ) of the stem cell niche (Daum et al., 2014; Yadav et al., 2010). Within the CZ, WUS is required for maintenance of stem cell identity. WUS is involved in a negative feedback loop with *CLAVATA3* (CLV3), which enables cell-cell communication between the CZ and the rib meristem (RM) to maintain a constant supply of stem cells for the SAM. CLV3 represses WUS expression, which confines WUS transcripts to the OC. Meanwhile, WUS positively regulates CLV3 expression in the CZ. (Schoof et al., 2000; Somssich et al., 2016).

When overexpressed in *Arabidopsis, At*WUS produced ectopic organogenesis which was further enhanced by co-overexpression of STM (Gallois et al., 2002). *At*WUS overexpression promoted somatic embryogenesis in *Coffea canephora* (Arroyo-Herrera et al., 2008) and cotton (*Gossypium hirsutum* L.) (Bouchabké-Coussa et al., 2013) as well as ectopic SAMs with malformed leaves in rice (Kamiya et al., 2003). However, both the coffee and cotton studies report an inability to regenerate whole plants from embryos overexpressing WUS, likely due to the deleterious effect of overexpression of WUS. Using an estradiol-inducible promoter, overexpression of *At*WUS allowed embryogenesis in an otherwise completely recalcitrant

Capsicum chinense (Solís-Ramos et al., 2009) and promoted shoot regeneration from root tips in tobacco (Rashid et al., 2007). Of significant interest to this thesis are the findings by B. Liu that show increased adventitious rooting and shoot formation in Chinese white poplar (*Populus tomentosa*) when overexpressing the *Populus trichocarpa* WOX11 (WUS-related homeobox) protein (B. Liu et al., 2014, 2018).

In addition to WUS, the AP2/ERF-related *BABY BOOM* (BBM) transcription factor also induces a switch from vegetative to embryonic growth. First identified in *Brassica napus,* BBM overexpression led to ectopic formation of somatic embryos and cotyledon-like structures on seedlings. Additionally, the ectopic embryos could produce plants in the absence of hormones (Boutilier et al., 2002). Later, it was discovered that overexpressing *At*BBM in the less-related species *Nicotiana tobaccum* did not lead to ectopic somatic embryogenesis. However, overexpression of a steroid-inducible *At*BBM-GR fusion led to ectopic shoot, root, and somtic embryos in hypocotyl explants in the presence of the inducing ligand dexamethasone (Srinivasan et al., 2006). Soybean BBM, *Gm*BBM, overexpression led to ectopic somatic embryos in *Arabidopsis* (El Ouakfaoui et al., 2010), while overexpression of oil palm (*Elaeis guineensis*) and dog rose (*Rosa canina*) BBM orthologs enhanced regeneration of explants *in vitro* without inducing ectopic spontaneous somatic embryogenesis (Morcillo et al., 2007; Yang et al., 2014), and transient expression of cocoa tree (*Theobroma cacao*) BBM, *Tc*BBM, increased embryogenic potential in both *T. cacao* and *Arabidopsis.* However, constitutive expression inhibited subsequent development (Florez et al., 2015). Contrastingly, overexpression of a camphor laurel (*Cinnamomum camphora*) BBM ortholog did not increase somatic embryogenesis or regeneration in *Arabidopsis*, although it did lead to small, rumpled, and variegated leaves (Liu et al., 2016).

There are several other DEV genes which have received less attention but have nonetheless made headway in plants which are difficult to regenerate. One of these genes is the RWP-RK protein RKD4, which is essential for the first asymmetrical division of the zygote which leads to the development of the embryo and suspensor cells (Jeong et al., 2011; Waki et al., 2011). Following these reports was a report from Indonesia of ectopic somatic embryogenesis in a recalcitrant hybrid *Phalaenopsis* orchid when *At*RDK4 was chemically induced (Mursyanti et al., 2016). First characterized in *Arabidopsis, LEAFY COTYLEDON1*

(LEC1) is involved in embryo maturation and can induce somatic embryogenesis when ectopically expressed (Lotan et al., 1998). There have been several attempts at using LEC1 as a DEV gene. Constitutively expressing the *Citrus sinensis* LEC1 paralog successfully induced somatic embryogenesis on previously-recalcitrant *Citrus* hypocotyl segments which gave rise to transgenic plants (Zhu et al., 2014). A similar study in Norway spruce (*Picea abies*) demonstrated that overexpressing the conifer LEC1-type gene *Pa*HAP3A did not give rise to ectopic somatic embryos in vegetative tissues but did lead to ectopic embryos on the surface of zygotic embryos (Uddenberg et al., 2016). Heterologous constitutive expression of *Latrix decidua* LEC1 in *Arabidopsis* led to malformed, chlorotic, and shrunken cotyledons in addition to low seed germination rates, demonstrating cross-species importance of LEC1 for embryo and cotyledon development (Rupps et al., 2016).

Many of the above mentioned reports of enhanced regeneration, whether embryonic or organogenic, also reported deleterious pleiotropy as a result of overexpressing DEV genes. There are a few strategies to overcome this, but all seek to limit DEV gene expression to a specific temporal or spatial range to allow for normal plant development. The strategies that have been employed to this end include inducible expression of the DEV gene through addition of a chemical ligand (removal of the ligand ceases expression), gene excision, use of a promoter which is temporally-limited, and using *Agrobacterium* to deliver the T-DNA transiently. Each strategy will be discussed along with its advantages and disadvantages.

Inducible expression of DEV genes

Constitutive and ubiquitous expression of DEV genes can result in deleterious effects on plant growth and development (Lowe et al., 2016; Maher et al., 2020). Tissue-specific promoters can confine the expression to specific tissues, but they may not allow for precise control of expression over time. Inducible expression allows for only conditional expression (or activation) of the gene of interest at the desired time. Several inducible expression systems have been applied to plants. Chemically-inducible systems include β-estradiol (Bruce et al., 2000; Zuo et al., 2000), ethanol (Caddick et al., 1998; Roslan et al., 2001), copper (Saijo & Nagasawa, 2014), and dexamethasone (Jasinski et al., 2005; Kirch et al., 2003). Environmental induction of expression may be done by using promoters which are responsive to specific abiotic stresses such as heat (Freeman et al., 2011; Shinmyo et al., 1998), cold (Kovalchuk et al., 2013), light

(Yumerefendi et al., 2015), and drought (Xue et al., 2011). However, inducible expression systems can also be 'leaky' (expressed without the chemical or stress) in certain plants and contexts (De Veylder et al., 2000; Omelina et al., 2022).

The glucocorticoid receptor (GR)-dexamethasone (DEX) system is a widely-used inducible gene activation system in plants. In this system, the ligand-binding domain of the rat GR is translationally fused with the plant protein of interest, and the fusion protein will be prevented from entering the nucleus by its association with heat-shock protein 90 (HSP90). Upon addition of the ligand DEX, the complex dissociates which enables translocation of the fusion protein to the nucleus (Picard et al., 1988; Schena et al., 1991). The first reported use of this inducible activation system with a DEV gene used the *Brassica napus* BBM-GR fusion, which promoted spontaneous shoot and root formation in tobacco in the presence of DEX (Srinivasan et al., 2006). Following this report, Heidmann et al. (2011) used the same construct to enhance the rate somatic embryogenesis in recalcitrant sweet pepper (*Capsicum annuum*) from 0% (*35S:GUS)* to 1%. Additionally, embryos recovered could be developed into fertile plants which could transmit the transgene to progeny.

Gene excision

In many crops, transgenes such as DEV genes can be removed via crossing and segregating away the DEV gene, this is not possible for clonal crops. This limitation necessitates the removal (termed 'excision') of the DEV gene following regeneration. Multiple excision systems have been tested in plants, including homologous recombination (Puchta, 2000; Zubko et al., 2000), transposition (X. Gao et al., 2015; Maeser & Kahmann, 1991), CRISPR/Cas-mediated excision (Sheva et al., 2020; Srivastava et al., 2017), and recombination. Multiple recombination systems such as the *Cre/lox* site-specific recombinase (SSR) from bacteriophage P1 (Hoess et al., 1982; Hoess & Abremski, 1985), R/RS from *Zygosaccharomyces rouxii* (Araki et al., 1985), and *Flp/frt* from brewer's yeast (Cox, 1983; Senecoff et al., 1985) have been used with varying degrees of success in plants. The *Cre/lox* SSR has many advantages over other excision systems because of its ease of use. In addition to delivering the *Cre* recombinase enzyme, the system requires only that the sequences to be excised be flanked by *Cre* recognition sites, termed 'loxP' sites. The 'floxed' (from 'lox-flanked') sequences will then be circularized and excised (Sternberg & Hamilton, 1981). *Cre/lox* excision in plants can be achieved in several

ways. Firstly, the floxed sequence can be stably transformed into plant cells, and following regeneration, the progeny plants can be stably re-transformed with a Cre-containing vector (Odell et al., 1990). Secondly, two separate vectors (one which contains the floxed sequence and a second which contains the Cre recombinase sequence) may be co-transformed at once. Following transformation, stably co-transformed plants will undergo Cre excision (Dale & Ow, 1991). In species where sexual-outcrossing is possible, two stably-transformed plants, one containing floxed sequence(s) and the other containing Cre, may be mated for excision to occur in progeny zygotes (Bayley et al., 1992). Sexual crossing works in the same fashion with the *Flp/frt* recombination system as well (Kerbach et al., 2005; Kilby et al., 1995).

1.7.3 Biological functions of GRF-GIF and use as a novel DEV gene

GROWTH REGULATING FACTOR (GRF) is a small, plant-specific transcription factor (TF) family. GRF transcription factors are found in nearly every species of plant, from single-celled algae to mono- and dicotyledonous plants (Kim, 2019). Since the initial discovery of *OsGRF1* in rice (van der Knaap et al., 2000), numerous studies have identified GRFs and their functions in *Arabidopsis* and crop species (rev. in Liebsch & Palatnik 2020). Characterized by conserved QLQ and WRC domains, GRFs are involved in both protein and DNA binding. GRF TFs are involved in transcriptional regulation of genes involved in central developmental processes including leaf, root and stem development, flower and seed formation, meristematic development (J. H. Kim & Lee, 2006) and modulation of growth processes during unfavorable environmental conditions (Heidel et al., 2004). GRF is post-transcriptionally regulated by microRNA396 in mature tissues and the stem cell niche of the root meristem (Omidbakhshfard et al., 2015). Additionally, GRF complexes with a transcriptional co-regulator, *GRF-INTERACTING FACTOR* (GIF). Due to their roles as master regulators of plant development, biotechnological and agricultural interest in GRF-GIF and miRNA396 has grown in recent years (J. H. Kim, 2019a).

Post-transcriptional regulation of GRF

Most GRF proteins are post-transcriptionally regulated by miRNA396, which has perfect complementarity with a conserved region near the WRC domain of GRF mRNA. The miRNA396 binds to GRF mRNAs, which leads to transcript cleavage or translational arrest. (Omidbakhshfard et al., 2015). During miRNA396 overexpression, a characteristic *grf* mutant

phenotype is observed (Rodriguez et al., 2015). Using this knowledge of the post-transcriptional regulation of GRF mRNAs, mutations can be made in the binding region of GRF that render the transcripts incompatible with miRNA396. These mutations result in higher expression of GRFs, which in turn leads to larger leaves and other organs (Liebsch & Palatnik, 2020). The same effect can be achieved by targeted miRNA396 gene knockout and the use of target-mimicry technology, where a non-coding RNA which binds to and deactivates miRNAs is constituently expressed (F. Gao et al., 2016). Additionally, some members of the *Arabidopsis* GRF family are found to be natively miRNA396 resistant and are instead regulated by the transcription factor *AUXIN RESPONSE FACTOR 2* (ARF2) (Beltramino et al., 2021). However, further study will need to elucidate whether this is a widely conserved mechanism or if it is specific to Brassicaceae.

Meristematic Specification

One of the primary functions of GRF is promoting cell division of transit-amplifying cells (TAC), which are the daughter cells of mersitematic stem cells (Ercoli et al., 2018; Rodriguez et al., 2015). GRF also promotes organogenesis by repressing *Knotted1-Like Homeobox (KNOX)* genes, which are responsible for maintaining the pool of stem cells inside the central zone (CZ) of the shoot apical meristem (SAM) by repressing organogenesis (Mayer et al., 1998). It has been proven experimentally in rice, barley, and *Arabidopsis* that GRF and GIF proteins bind to the promoter region of *KNOX* genes and repress their transcription. RNA interference of rice GRF3, 4, and 5 resulted in significantly higher expression levels of the *KNOX* gene *OsKN2* (the rice homolog of *Arabidopsis KNAT2*) (Kuijt et al., 2014). The *Arabidopsis* quadruple mutant (*grf1/2/3/4)* had reduced leaf size, fewer leaf cells, fused cotyledons, and displayed remarkable similarity to the *shoot meristemless (stm)* mutant phenotype. In accordance with the *stm* phenotype, the quadruple mutant also completely lacked a SAM (Kim & Lee, 2006). Thus, GRF-GIF plays a vital role in meristematic specification through its interaction with *STM* and central meristem development processes.

Stem, Leaf, and Root Growth

In addition to their roles in meristematic development, GRF and GIF play important roles as positive and negative regulators of plant growth. GRF and GIF expression are mostly associated with proliferating cells in numerous organs (Ercoli et al., 2018; Rodriguez et al., 2015,

2016), and both *grf* and *gif* mutants develop small leaves with fewer cells ((Ercoli et al., 2018; J. H. Kim & Kende, 2004). Combining *grf* and *gif* mutations synergistically reduces leaf size, whereas combining overexpressed GRF and GIF synergistically increases leaf growth (Debernardi et al., 2014). Increases in leaf size and plant height have also been observed in CRISPR/Cas9 knockouts of miRNA396 (Hou et al., 2019). GIF interacts with both GRF proteins and the SWItch/Sucrose Non-Fermentable (SWI/SNF) ATPase chromatin-remodeling machinery of *BRAHMA* and *SPLAYED* (Debernardi et al., 2014; Nelissen et al., 2015; Vercruyssen et al., 2014). This indicates that there is a formation of a physical complex between GRF and GIF that transcriptionally promotes leaf cell proliferation through chromatin remodeling (Debernardi et al., 2014; Vercruyssen et al., 2014). Additionally, GIFs downregulate transcription of *PLETHORA* (*PLT*) transcription factors, perhaps in concert with GRFs. During embryo development, *gif* mutants produce only roots instead of cotyledons (Ercoli et al., 2018).

Application of GRF-GIF as a DEV gene

As a master regulator of multiple developmental processes, there may be multiple biotechnology applications for the GRF-GIF/miRNA396 module. Due to the intermediate position of this transcription module in the greater network of growth and development genes, downstream responses can be elicited within the framework of a single developmental context without upsetting the balance of the entire upstream development pathway. The ability to mutate individual GRF genes to be either partially or fully resistant to miRNA396 binding provides a toolkit for fine-tuning gene expression. Depending on the number of mismatches in the complementarity between miRNA396 and GRF mRNAs, the GRF gene can be partially or fully silenced. This is important because knocking out miRNA396 or drastically reducing its transcripts can lead to deleterious pleiotropic effects throughout the developmental cycle (Debernardi et al., 2012).

Several publications have reported that GRF-GIF overexpression can be used to enhance transformation efficiency in a wide range of economically significant yet recalcitrant crop species including wheat (*Triticum spp.*), hemp (*Cannabis sativa*), and watermelon (*Citrullus lanatus*; Debernardi et al., 2020; Feng et al., 2021; Kong et al., 2020; Zhang et al., 2021). By overexpressing a fused chimera comprised of wheat *TaGRF4-GIF1* in transformed embryos of a recalcitrant tetraploid wheat line, transformation efficiency increased by 7.8-fold compared to

the empty vector control. Additionally, the chimera sped up the regeneration process by more than one month. Overexpressing *TaGRF4* or *TaGIF1* alone showed a much weaker responses than the fused chimera of the two proteins. Similarly, the overexpression of other fused *Ta*GRF-GIF proteins did not perform as well as *Ta*GRF4-GIF1, including a *Ta*GRF5-GIF1 chimera which also showed a significant increase in transformation efficiency. The *Ta*GRF4-GIF1 chimera also enhanced regeneration of transformed embryos of recalcitrant commercial lines of hexaploid bread wheat (*Triticum aestivum*), triticale (× *Triticosecale*), and durum wheat (*Triticum turgidum*). To test whether synthetic cytokinins were required for shoot regeneration in nascent meristems expressing *Ta*GRF4-GIF1, another experiment was conducted with two groups, one that contained the fused *Ta*GRF4-GIF1 chimera and one that did not. Even in the total absence of cytokinin, a 6.8x increase in regeneration efficiency was observed in the *Ta*GRF4-GIF1 group. (Debernardi et al., 2020). These results are extremely promising for the future of wheat transformation and the transformation of other economically significant and recalcitrant commercial monocot genotypes. There were no published reports of GRF4-GIF1 failing to improve regeneration efficiencies in monocot species.

In addition to testing *GRF* overexpression in wheat, Debernardi et al. tested the GRF4-GIF1 chimera in two dicot species, grape and citrus. Grape and citrus homologs were selected based on their homology to wheat GRF4 and GIF1 and were similarly fused to form a chimeric protein. In separate experiments, citrus epicotyls were transformed with both the grape and citrus chimeras, resulting in a 4.7x increase in regeneration efficiency compared to the empty vector for the citrus homolog, and a similar increase was observed using the grape homolog. A miRNA396-resistant GRF4 grape homolog was produced using silent mutations in the miRNA396 binding site on GRF4. A 7.4x increase in transformation efficiency was observed for citrus epicotyls overexpressing this protein, although further optimization will be required because some transformants generated large non-regenerable calli but could not produce shoots.

Another study using *Arabidopsis* GRF5 *(At*GRF5*)* reported transformation improvement. Sugar beet explants transformed with 35S:*At*GRF5 produced a larger number of shoots compared to the control. Additionally, a higher percentage of shoots recovered from the *At*GRF5 treatment group were transgenic. The total percentage of shoots that were transgenic for all experiments was 97.5%, leading to an overall transformation efficiency rate of 19.6% and a 6x increase

compared to the control group. Strikingly, there were no observed phenotypic differences between the two treatment groups, suggesting that there are limited pleiotropic effects from overexpression of GRF5. These results also indicate that GRF5 can improve the switch between callus and organogenesis phases in *in vitro* tissue culture conditions. Interestingly, when the same experiment was repeated using the closest sugar beet GRF5 ortholog, the transformation efficiency was still increased, however the improvement over the control group was statistically nonsignificant ($P > 0.05$). It is worth noting that *At*GRF5 lacks a miRNA396 binding site, and its transcriptional regulation mechanism remains largely unknown (Beltramino et al., 2021).

Although investigation of GRF overexpression in other recalcitrant species is ongoing, there is a growing body of evidence that overexpressing GRF singularly and with its cofactor GIF has promise in shortening the length of *in vitro* transformation experiments and increasing transformation efficiency. Additionally, there appear to be limited pleiotropic effects of overexpressing these transcription factors, owing to the post-transcriptional control by miRNA396. This hypothesis is substantiated by the evidence that while miRNA396-resistant constructs generated large calli, some were unable to form shoots. Some researchers think that an inducible activation system, such as a mammalian glucocorticoid receptor fusion with GRF-GIF which inactivates once dexamethasone is removed, may allow non-regenerable calli to produce shoots (Debernardi et al., 2020).

Chapter 2: Using GRF-GIF to promote regeneration in *Populus* and *Eucalyptus*

2.1 Introduction

Increasing demand for forest products threatens forest biodiversity globally (Willis, 2003). Approximately one third of global timber supply originates in forest tree plantations, while plantations use only 5% of global forest land (FAO, 2010). Poplar and eucalypt hybrids are popular plantation trees due to their fast growth rate and fiber qualities (CIRAD-FRA et al., 2018; Isebrands & Richardson, 2013). The incorporation of desirable traits to elite poplar or eucalypt cultivars by traditional breeding and recurrent selection can take decades and can 'carry over' undesirable linked alleles in the process. However, genetic engineering (GE) can accomplish the same task in a single generation without the risk of 'carrying over' unwanted alleles (Ahuja, 2011). In addition, traits external to the sexual gene pool can be introduced, such as new forms of biotic and abiotic stress resistance.

Outside of a few regenerable genotypes, *in vitro* regeneration of transgenic shoots remains challenging in most poplar and eucalypt genotypes As global demand for timber increases, there is a strong need for improved regeneration systems which will enable genetic engineering in these trees (Nagle et al., 2018). Overexpressing a chimeric protein consisting of *GROWTH-REGULATING FACTOR 4* (GRF4) and *GRF INTERACTING FACTOR 1* (GIF1) transcription factors has enhanced transgenic shoot regeneration in several diverse species of crop plants including one woody species – citrange (*Citrus x insitorumi*) (Debernardi et al., 2020b). Here, I report the methods and results of overexpressing two GRF4-GIF1 transcription factor chimeras in two distinct hybrid poplar genotypes – a miRNA396-resistant chimera derived from *Citrus* and a miRNA396-sensitive chimera derived from *Populus*. Supplementary poplar and eucalypt experiments testing GRF-GIF chimeras from *Citrus* with varying levels of miRNA396 sensitivity and a chimera from *Vitis vinifera* expressed constitutively and in a steroid-activated form are located in Appendices B-D. See Appendix Table A.1 for comprehensive details about each construct.

2.1.1 Hypothesis

Our primary hypothesis was that GRF4-GIF1 overexpression would increase the number of transgenic shoots produced by two hybrid poplar genotypes *in vitro*. The number of shoots is

defined here as shoots/Petri dish (a.k.a. ‘plate’), and plates were standardized with roughly equal numbers of explants.

2.2 Materials and Methods

I used a synthetic miRNA396-resistant *Citrus x clementina* GRF4-GIF1 gene chimera (*Citrus* rGRF4-GIF1) given to our lab by the Dubcovsky lab at the University of California, Davis; it was based on the annotated genome of *Citrus* x *clementina* (Debernardi et al., 2020; Wu et al., 2014). The GRF4 gene sequence had a four base pair (bp) mutation introduced to eliminate miRNA-396 binding (Figure 2.1) (Debernardi et al., 2020; Rodriguez et al., 2010).

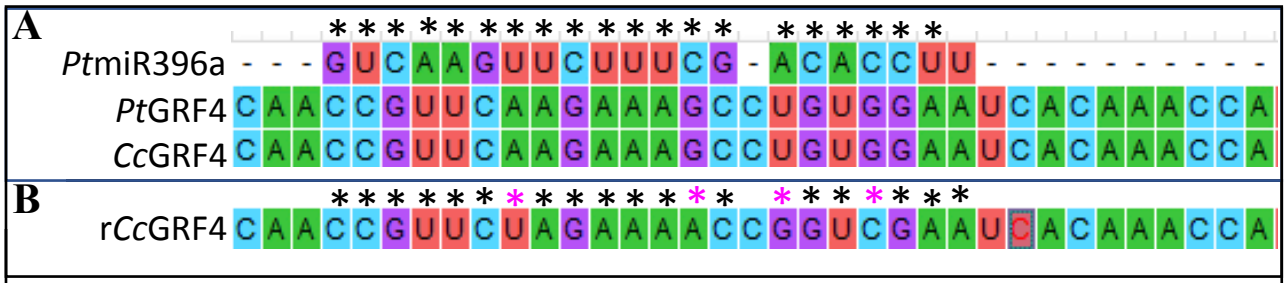

Figure 2.1: Alignment of miRNA396 and GRFs used in this project. (A) miRNA396 from *Populus trichocarpa* (*Pt*miR396a) and GRF4 mRNAs from *P. trichocarpa* (*Pt*GRF4) and *Citrus x clementina* (*Cc*GRF4). Asterisk (*****) indicates miRNA complementarity with GRF mRNAs. **(B)** miRNA396-resistant GRF4 (r*Cc*GRF4) from *C. x clementina*. Mutations are highlighted with magenta asterisks (*).

In addition to the *Citrus* rGRF4-GIF1 chimera, I generated a *Populus trichocarpa* synthetic GRF4-GIF1 chimera (*Pt*GRF-GIF) following a phylogenetic analysis to find the closest *P. trichocarpa* orthologs to *Citrus* and *Vitis* GRF4 and GIF1. All GRF-GIF chimeras were ligated into binary vectors containing the dsRed2 red fluorescent protein (Nishizawa et al., 2006) and a hygromycin B selectable marker, *hpt* (Blochlinger & Diggelmann, 1984). I then used *Agrobacterium*-mediated transformation (AMT) with *A. tumafaciens* strain ‘AGL1’ to introduce the chimeras to *in vitro*-grown explant tissues. After transformation, explants were evaluated for shoot regeneration at pre-determined time points (detailed below). Additional observations, including plant morphologies, were recorded with high-resolution brightfield and fluorescent microscope images.

2.2.1 *Populus* and *Eucalyptus* GRF & GIF ortholog selection

Construction of phylogenetic trees and analysis of tissue-specific expression patterns

For identification of *Populus trichocarpa* and *Eucalyptus grandis* orthologs to *Citrus* and *Vitis* GRF4 and GIF1 (Debernardi et al., 2020), two phylogenetic trees were constructed using all annotated GRF and GIF proteins from *Vitis vinifera, Citrus sinensis, Populus trichocarpa, Eucalyptus grandis,* and outgroup species *Oryza sativa spp. japonica.* Complete sets of GRF protein sequences from each species were downloaded from the public database PlantTFDB (Jin et al., 2017). To find GIF protein sequences, the SSXT domain of the *Arabidopsis* GIF1 protein was used as a seed sequence to search the National Center for Biotechnology Information database using the basic alignment search tool for proteins (BLASTP) (NCBI Resource Coordinators, 2016). To ensure all putative GIF genes were included, the protein sequences were further confirmed through the Phytozome website (Goodstein et al., 2012).

Amino acid sequences of the selected GRF and GIF proteins were imported into the software MEGA7 (Kumar et al., 2016) for alignment and construction of maximum likelihood phylogenies. Alignment was done using the ClustalW algorithm (Thompson et al., 1994). The evolutionary history of GRF and GIF proteins was inferred by using the Maximum Likelihood method based on the JTT matrix-based model (Jones et al., 1992). A bootstrap consensus tree (Figure 2.5) inferred from 1,000 replicates was constructed and branches corresponding to partitions reproduced in less than 50% of bootstrap replicates were collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) are shown next to the branches (Felsenstein, 1985). Initial trees for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using a JTT model, and then selecting the topology with superior log likelihood value. All positions containing gaps and missing data were eliminated. Genes corresponding to the GRF and GIF proteins selected from phylogenetic analysis were queried on the PlantGenIE and EucGenIE public resources (Christie et al., 2021; Sundell et al., 2015) to examine their organism-wide expression patterns from RNA-seq experimental data.

Cloning and synthesis of GRF and GIF genes

Forward and reverse PCR primers were designed for cloning the selected *P. trichocarpa* and *E. grandis* GRF and GIF genes (see Appendix Tables A.1 & A.2 and Figures A.2 & A.4). Primers were designed with flanking restriction enzyme sites for downstream restriction enzyme cloning, and GRF forward primers included a 5' UTR sequence from *Arabidopsis* which enhances translational efficiency of transgenes (Y. Kim et al., 2014). RNA was extracted from leaves and stems of *in vitro*-grown plantlets of *P. trichocarpa* clone 'Nisqually-1.' Extracted RNA was converted to cDNA using the iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, California, USA). The cDNA concentration and purity were determined from the 260/280 nm ratio using a NanoDrop 2000 spectrophotometer (Thermo Fischer Scientific, Waltham, Massachusetts, USA). Ultimately, GRF4 and GIF1 genes could not be cloned from cDNA, perhaps because of low expression of these genes in stems and mature leaves (section 2.3.1). Following the failed cloning attempts, CDSs were synthesized by a contracted company (Integrated DNA Technologies, San Diego, California, USA).

2.2.2 Vector construction

Citrus and *Vitis* GRF4-GIF1 sequences were amplified from bacterial plasmid DNA, while *Populus* GRF4-GIF1 and *Eucalyptus* GRF5-GIF1 sequences were amplified from synthetic gene fragments via PCR (Mullis et al., 1986). The stop codon was removed from the GRF coding sequences and replaced with four alanine codons before the methionine codon of GIF1, which yielded a fused GRF-GIF chimera. GRF-GIF PCR amplicons were ligated into a Golden Gate entry vector downstream of either a double or single 35S CaMV promoter (Covey & Hull, 1981) (Figure 2.2) using Instant Sticky-end Ligase Master Mix (New England Biolabs, Ipswich, Massachusetts, USA) at 25°C and immediately mobilized to *E. coli* via heat-shock transformation (Chung et al., 1989). Ligations were confirmed by enzymatic digestion of purified plasmid DNA and gel electrophoresis (Arber, 1978; Tiselius, 1937). GRF-GIF and dsRed2 entry vectors were then incorporated into a customized pTRANS220 transformation vector (Čermák et al., 2017) via Golden Gate assembly (Engler et al., 2008, 2009).

A Bio-Rad C1000 thermocycler (Bio-Rad Laboratories, Hercules, California, USA) was used for obtaining the PCR amplicons from bacterial DNA and synthetic gene fragments. PCR reactions were performed using Q5 High-Fidelity DNA polymerase (New England Biolabs,

Ipswich, Massachusetts, USA), manufacturer-supplied Q5 buffer, a 2.5mM mixture of dNTPs, oligonucleotide primer sets and template DNA. Annealing temperature was set to 55°C.

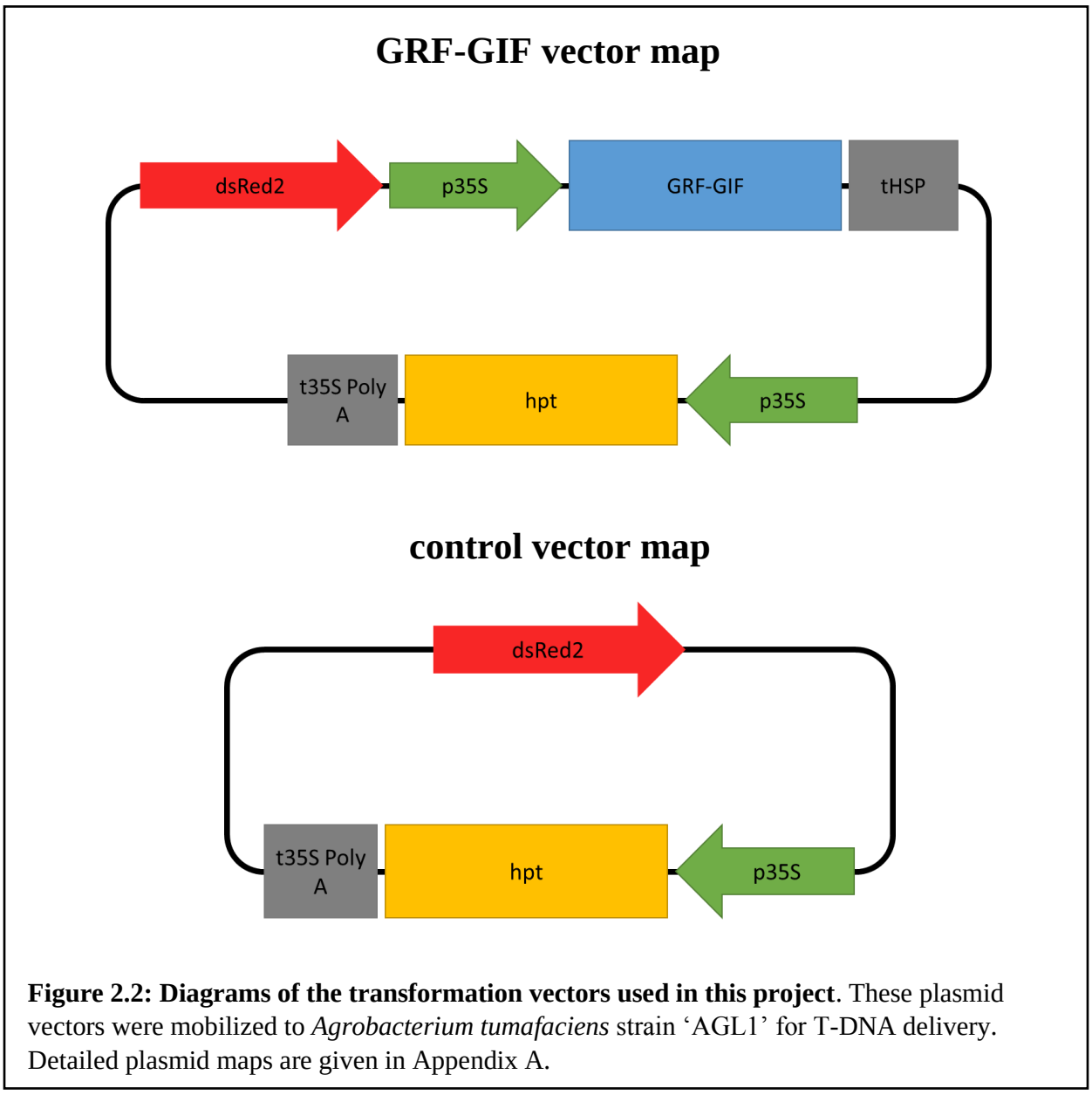

Figure 2.2: Diagrams of the transformation vectors used in this project. These plasmid vectors were mobilized to *Agrobacterium tumafaciens* strain 'AGL1' for T-DNA delivery. Detailed plasmid maps are given in Appendix A.

2.2.3 Plant material and transformation

In this study, I transformed hybrid poplar clones 717-1B4 (*P. tremula x P. alba*) and 353-53 (*P. tremula x P. tremuloides*). For experiments using *Citrus* rGRF-GIF, inter-nodal stem segments, petioles, and leaf disks from *in vitro* grown plantlets of uniform age and health served as explants. Experiments using *Pt*GRF-GIF used only inter-nodal stem segments. Explants were

inoculated by micro-wounding and swirling with a liquid *A. tumafaciens* induction culture (OD 0.6). *A. tumafaciens* strain AGL1 was used for all experiments. Explants and bacteria were co-cultivated on callus induction medium (CIM) for 48h in the dark. After this period, explants were washed with sterile ddH_2O and antibiotics timentin and cefotaxime (2.9×10^{-7} mg/l) to kill any remaining bacteria. After washing, the explants were placed on CIM containing timentin, cefotaxime, and hygromycin-B for 3 weeks and placed in darkness, after which they were subcultured onto shoot induction media (SIM) containing the same antibiotics and placed in full-strength light (16h light/8h dark) for 3-4 weeks. After shoot induction, a subset of explants were moved to elongation medium (EM) and/or rooting medium for $\geq$ 21 days. A full protocol can be found in the methods of Filichkin et al., (2006).

2.2.4 Analysis of *in vitro* regeneration by visual scoring

All explants in the *Citrus* rGRF-GIF experiments were placed on plates with 20 evenly-spaced explants (a 20-explant grid) and explants in the *Populus* GRF-GIF experiments were placed on 12-explant grid plates. In the *Citrus* rGRF-GIF experiments, leaf and stem (which included petioles in analysis) explants were kept on separate plates for analysis. Transgenic shoot regeneration was quantified using the dsRed2 fluorescent reporter provided to us by Corteva Agriscience (Johnston, Iowa, USA). Explants were evaluated for fluorescent shoot regeneration at 42-49 days post-transformation (PT) using a Nikon SMZ25 stereomicroscope (Nikon Instruments Inc., Tokyo, Japan) and an X-Cite epifluorescence LED illuminator (Excelitas Technologies Corp., Waltham, Massachusetts, USA). Shoot regeneration and ectopic root formation were recorded as a binary score (0 or 1) for each explant on the basis of absence or presence of at least one fluorescent transgenic shoot. Ectopic roots on leaf disk explants were scored in the same manner but without epifluorescence, so the rates represent total root regeneration (transgenic + escape).

2.2.5 Analysis of *in vitro* regeneration by GMOdetector

Fluorescent callus data was collected via a high-throughput phenomics pipeline 42-49 days PT. First, red-green-blue (RGB) and hyperspectral images of regenerating tissue in petri dishes were produced using a MacroPhor Array (Middleton Spectral Vision, Middleton, Wisconsin, USA). Next, segmentation of tissue types (callus, shoot, and stem) using RGB

images was performed using a Semantic-Guided Interactive Object Segmentation (SGIOS) model trained as described in previous work (Yuan et al., 2022). SGIOS could only be applied to stem and petiole explants, so callus data was not collected for leaf disk explants. Hyperspectral images were analyzed using CubeGLM (Python; https://github.com/naglemi/gmodetector_py.git) and then cross-referenced with RGB images using Python and R scripts to quantify reporter protein signals in specific tissues (e.g. callus, shoot) and classify tissues as transgenic or not (https://github.com/naglemi/GMOnotebook).

2.2.6 Statistical analysis

Statistical analyses were conducted using R software version 4.0.0 (R Core Team, 2019). Each experiment was first graphically examined using scatterplots to check for the presence of outliers which might have arisen from incorrectly recorded data or highly unusual plates. Scatterplots were made using the ggplot2 package (Wickham, 2016). Additionally, a generalized linear model was fit to each dataset, and Cook's distance was calculated from this model to detect influential outliers (>0.5) (Cook, 2000). During analyses of this study, only one influential outlier was identified and removed (Figure A.2). Cook's distance was calculated using the 'car' package (Weisberg & Fox, 2019). Differences in treatment group means were visualized using bar plots and error bars calculated using the standard error for each treatment group. Bar plots and error bars were manually constructed in Microsoft Excel (Microsoft Corporation, 2018). Figure 2.3 contains a schematic diagram of the data analysis process.

Due to the presence of fixed variables such as the treatment group (control or GRF-GIF) and the random error variable 'Replicate,' which captures the unaccounted-for variation between replicates, I decided that a mixed-effects model would work best for our analyses. However, after failing to meet the assumptions of the linear mixed-effects model (LMM), namely homoscedasticity and normality of distribution, all callus and shoot regeneration data was analyzed using a binomial generalized linear mixed-effects model (GLMM), which collapses all non-zero values to '1' and zero values as '0.' Only ectopic root formation data from 717-1B4 leaf disk explants was analyzed using an LMM. The two poplar genotypes were analyzed separately due to their disparate responses but compared using a Chi-squared test of independence where each plate was considered an independent observation.

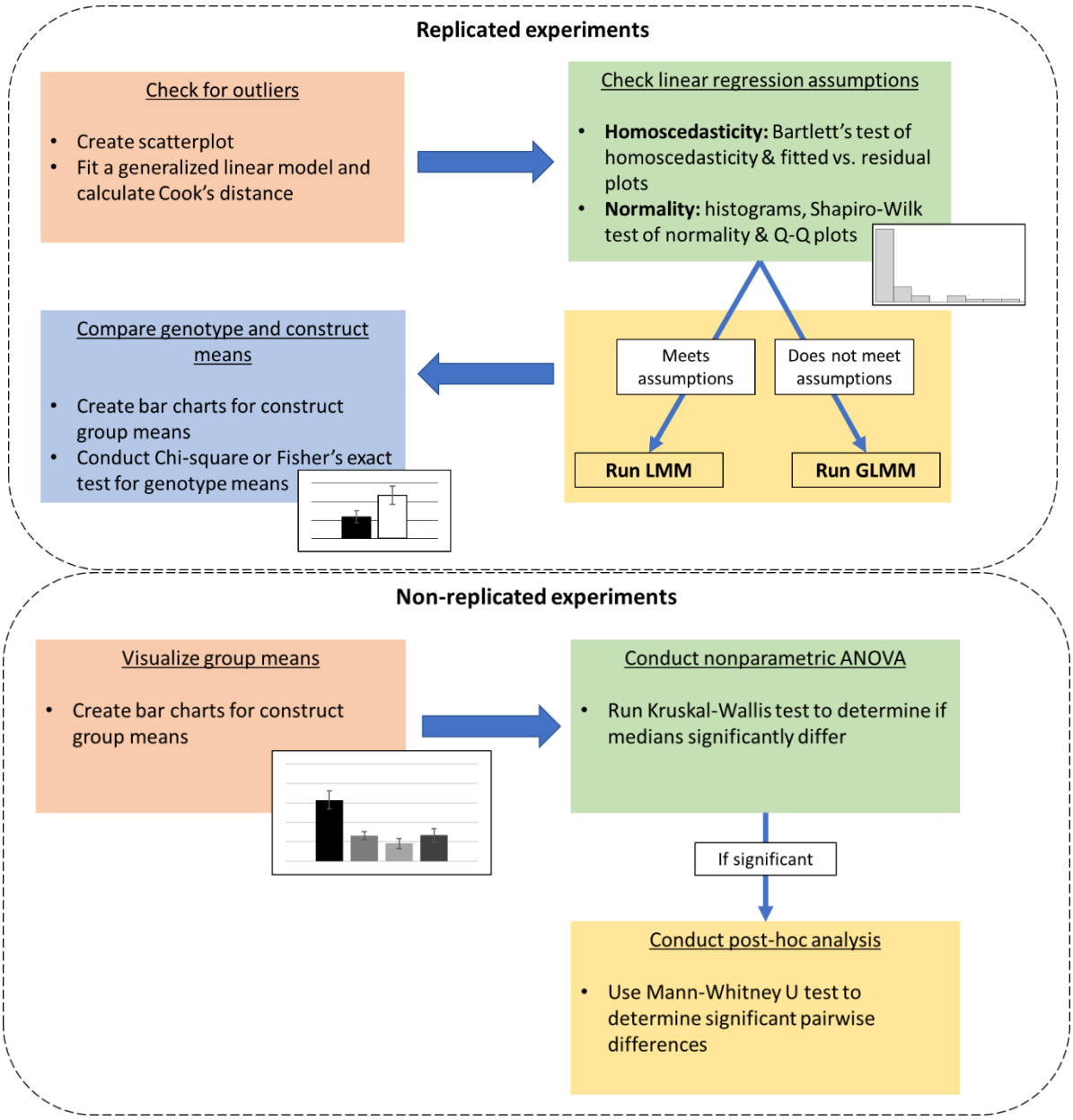

Figure 2.3: Order and logic of data and statistical analyses. LMM = linear mixed-effects model. GLMM = binomial generalized linear mixed-effects model.

Homoscedasticity and normality of distribution were checked using Bartlett's test (Bartlett, 1937) and the Shapiro-Wilk test, respectfully (Shapiro & Wilk, 1965). If the dataset passed the Shapiro-Wilk test ($P \geq 0.05$), a Q-Q plot was made to graphically examine normality. Explanatory variables for construct and number of explants/plate ('Num_explant') were treated as fixed variables, and replicate was treated as a random variable due to the unaccounted-for variation between replicates. While 92% of plates in the *Citrus* GRF-GIF experiment had 17-20

explants and 89% of plates in the *Pt*GRF-GIF experiment had exactly 12 explants, plates with fewer than 50% of the expected number of explants (either 12 or 20) were not included in the statistical analysis.

Binomial generalized linear mixed-effects model

Due to heterogeneous variance and non-normal distribution (Appendix Figure A.4) of the shoot regeneration data, statistical analyses of transgenic callus and shoot data were conducted using a binomial generalized linear mixed-effects model (GLMM), a function of the 'lme4' package in R. Binomial GLMMs have more relaxed assumptions than traditional linear mixed effects models and do not require homogeneity of variance or normally-distributed data because of the binomial distribution. The only assumptions are that data is both independent and random. In the analyses, plate means were treated as independent data points. The response variable was the binary transgenic shoot regeneration variable. The explanatory variables were the treatment (control or GRF-GIF), number of explants per plate, and replicate (i.e., independent experimental studies over time, as a random variable). An additional explanatory variable for tissue type (leaf or stem) was added to the *Citrus* rGRF-GIF model. The two experiments (*Citrus* and *Pt*GRF-GIF) were directly compared using a GLMM which included an additional variable for the week of regeneration analysis (either 6 or 7).

GLMMs used in this study:

Analysis of binary shoot regeneration per plate in the *Citrus* experiment:

- Model (GLMM) = binary regeneration ~ Construct + Number_explant + (Replicate) + Tissue

Analysis of binary shoot regeneration per plate in the Populus experiment:

- Model (GLMM) = binary regeneration ~ Construct + Number_explant + (Replicate)

Analysis of binary shoot regeneration per plate comparing the *Citrus* and *Populus* GRF-GIF experiments:

- Model (GLMM) = binary regeneration ~ Construct + Number_explant + (Replicate) + Tissue + Week

Linear mixed-effects model

Ectopic root formation data from 717-1B4 leaf disks was analyzed using a linear mixed-effects model, a function of the 'lme4' package in R (Bates et al., 2015). Subsequent p-values were calculated using the 'lmerTest' package (Kuznetsova et al., 2017), which uses Satterthwaite's degrees of freedom method to approximate the degrees of freedom (Satterthwaite, 1946).

LMM used for ectopic root regeneration on 717-1B4 leaf disks:

Model (LMM) = Root_count ~ Construct + Number_explant + (Replicate)

Chi-squared & Fisher's exact tests

For testing what effect genotype x treatment had on transgenic shoot regeneration, a Pearson's Chi-squared test of independence (Pearson, 1900) was used with Yates' continuity correction (Yates, 1934) with 1 degree of freedom. For Chi-squared analysis, at least 80% of the expected frequencies must exceed 5 and all the expected frequencies must exceed 1, which were met for our data (Appendix Figure A.2). Expected frequencies and Chi-squared tests were conducted in R using the base R function 'chisq.test' (R Core Team, 2019). When expected frequencies did not meet the minimum required for a Chi-squared test, a Fisher's exact test was used instead (Bower, 2003; Fisher, 1922). The R function fisher.test was used to conduct analyses (R Core Team, 2019).

Kruskal-Wallis nonparametric ANOVA

Due to small their sample sizes and heteroscedasticity, non-replicated experiments (Appendices B-D) were analyzed using the Kruskal-Wallis rank sum test, which is a non-parametric alternative to one-way ANOVA (Kruskal & Wallis, 1952). Kruskal-Wallis has relaxed sample-size requirements compared to most forms of linear regression, which is typically

not reliable when used for heteroscedastic datasets where n < 25 (Jenkins and Quintana-Ascencio, 2020; Meyer and Seaman, 2013). If Kruskal-Wallis indicated a significant difference between groups ($P < 0.05$), post-hoc analysis was conducted to determine which of the groups had significantly different transgenic shoot regeneration. Post-hoc analysis was conducted using the pairwise Wilcoxon rank sum test with corrections for multiple comparisons (Mann & Whitney, 1947).

2.3 Results

2.3.1 Phylogeny and tissue-specific expression patterns of *Populus* and *Eucalyptus* GRF & GIF genes

Phylogenetic analysis revealed unambiguous *P. trichocarpa* and *E. grandis* GIF1 orthologs which share close homology with GIF1 from both *Citrus sinensis* and *Vitis vinifera.* From *P. trichocarpa,* GIF1 ortholog Potri.019G013100 (*Pt*GIF1) was selected, and from *E. grandis,* GIF1 ortholog Eucgr.B02127 (*Eg*GIF1) was selected. There were two *P. trichocarpa* GRF proteins which appeared orthologous to *Citrus* and *Vitis* GRF4, Potri.013G077500 (*Pt*GRF4-1) and Potri.019G042300 (*Pt*GRF4-2). Due to the ambiguity, I examined organism-scale expression patterns using the PlantGenIE public resource (Sundell et al., 2015). Phylogenetic analysis revealed no close ortholog to *Citrus* or *Vitis* GRF4 in *E. grandis.* However, the closest GRF protein was Eucgr.F04420 (Figure 2.5), which is annotated as *E. grandis* GRF5 (*Eg*GRF5). When its amino acid sequence was blasted against the *Arabidopsis thaliana* proteome, *Eg*GRF5 was 66% identical to the *At*GRF5 used to enhance regeneration in a previous report (Kong et al., 2020). A protein alignment of GRF4 and GRF5 proteins used in this analysis can be found in Appendix Figure A.5. A species-level phylogeny of all of the species used in the GRF and GIF phylogenetic analyses, plus *Arabidopsis,* can be found in Figure 2.4.

Organism-level expression patterns of *Pt*GRF4-1, *Pt*GRF4-2, and *Pt*GIF1 revealed that both *Pt*GRF4-1 and *Pt*GIF1 are expressed most highly in actively dividing tissues such as young expanding leaves, phloem, dormant and pre-chilling buds, and dormant catkins (Figure 2.7). Curiously, absolute expression in log2(transcripts per million + 1) of *Pt*GRF4-2 was 3.2x lower in bud tissue than *Pt*GRF4-1 (1.9 vs 6.1) and was not expressed in cambium at all. Based on this result, *Pt*GRF4-1 was selected over *Pt*GRF4-2.

Expression patterns of *Eg*GRF5 and *Eg*GIF1 revealed a similar expression pattern to that of the poplar genes. Both genes are upregulated in new growth, including shoot tips and young leaves. However, *Eg*GIF1 appears to be expressed more broadly than *Eg*GRF5 (Figure 2.6). Based on the results of the phylogenetic analysis and expression data, *Pt*GRF4-1, *Pt*GIF1, *Eg*GRF5, and *Eg*GIF1 were selected for cloning.

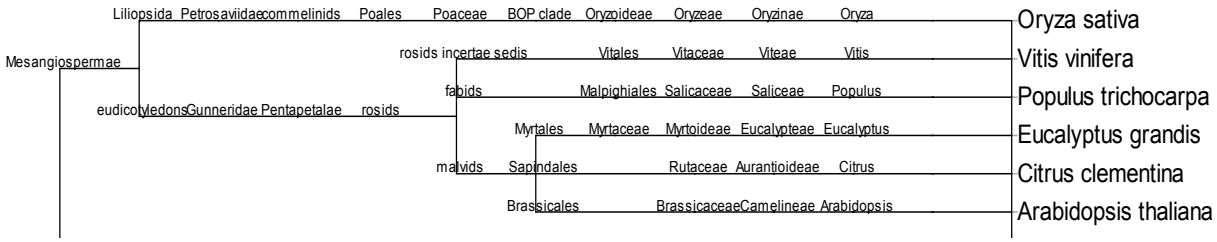

Figure 2.4: Evolutionary relationship between species. The evolutionary relationship between all species used in the phylogenetic analyses plus *Arabidopsis thaliana* including families, orders, and clades. This tree was constructed using the iTOL online resource (Letunic and Bork, 2021).

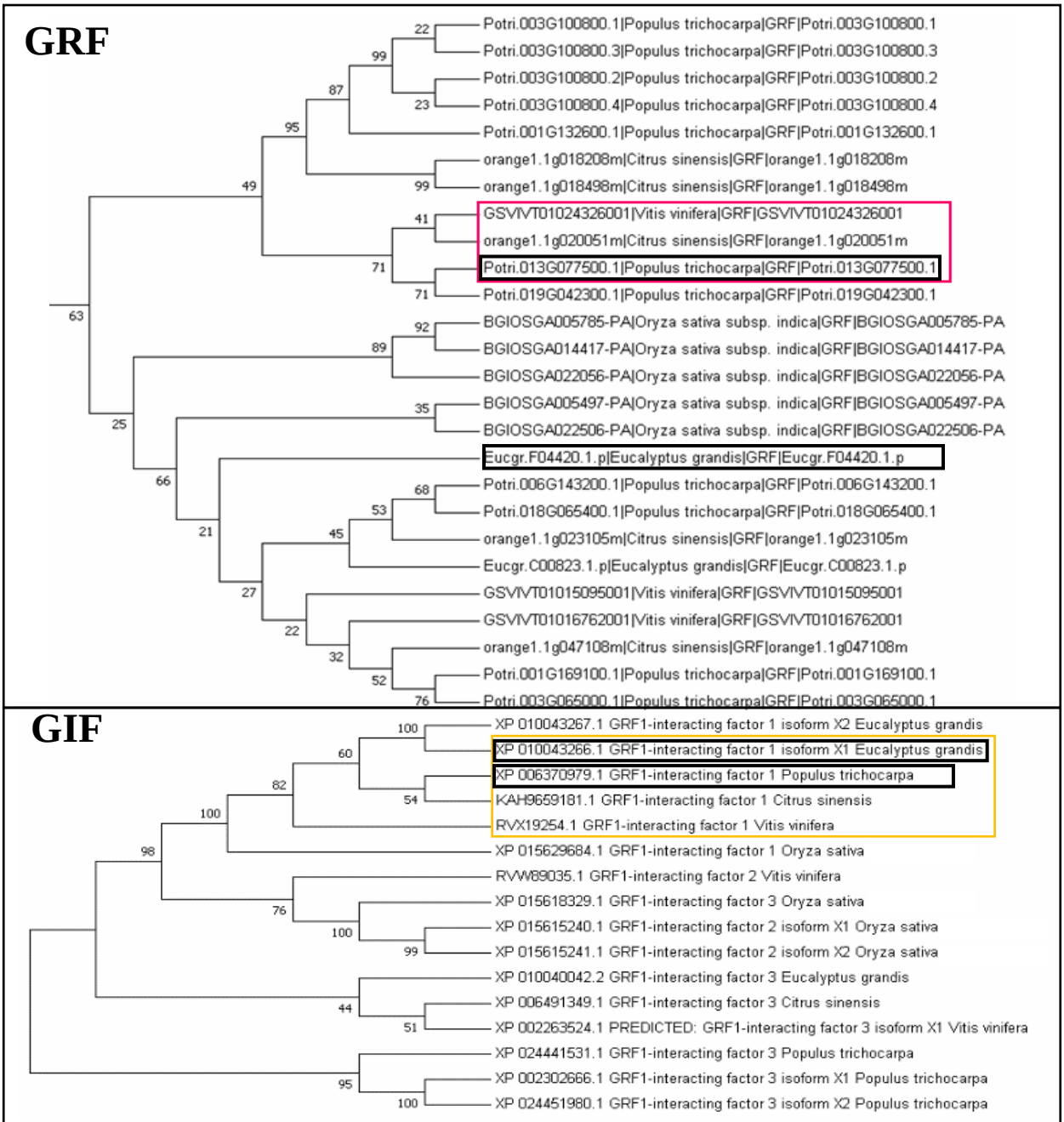

Figure 2.5: Bootstrap consensus trees showing evolutionary history of *GRF* & *GIF* proteins from *Vitis vinifera, Citrus sinensis, Populus trichocarpa, Eucalyptus grandis,* and *Oryza sativa.* The trees inferred from 1,000 replicates were constructed and branches corresponding to partitions reproduced in less than 50% of bootstrap replicates were collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). *GRF4-like* proteins from *Citrus, Vitis,* and *Populus* are highlighted in the magenta box and *GIF1-like* proteins in the yellow box. Selected *Populus GRF4-like, Eucalyptus GRF5-like,* and *GIF1-like* proteins used in constructs are highlighted in black boxes.

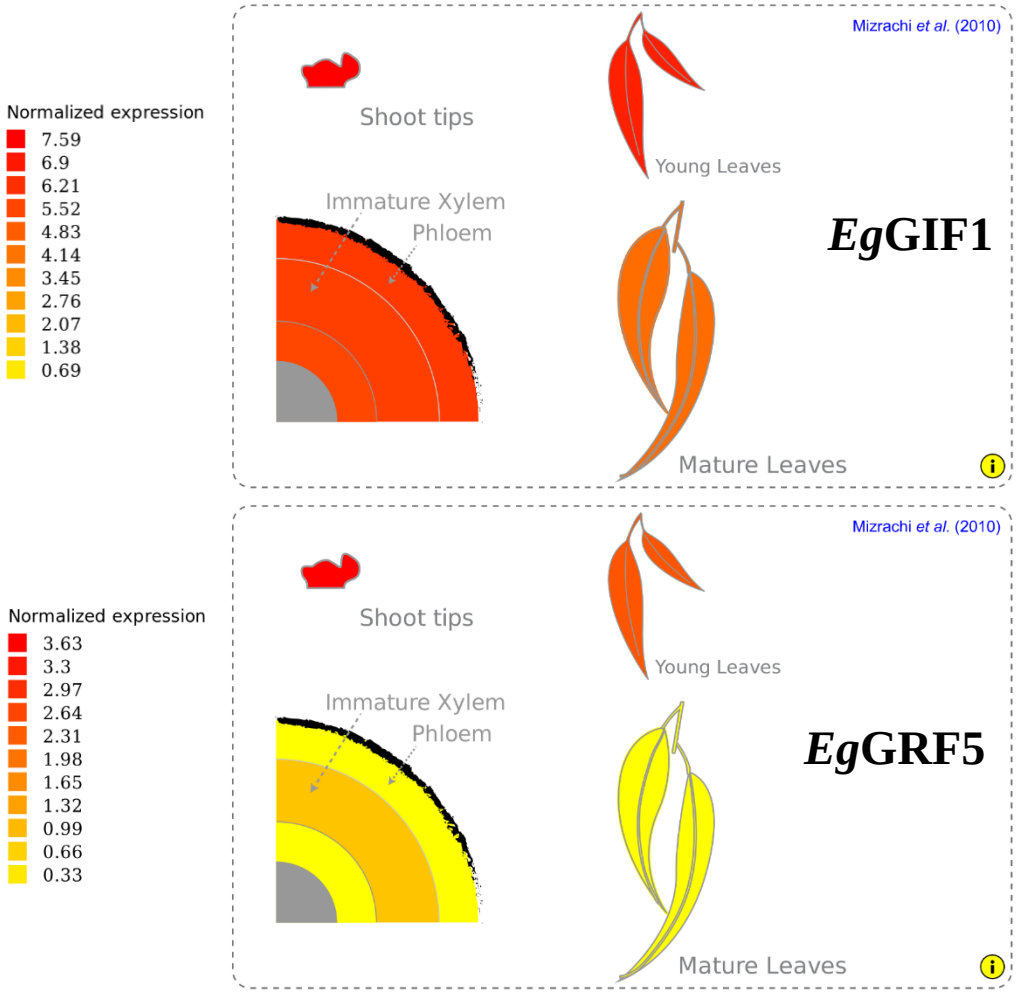

Figure 2.6: Absolute expression heatmap of GRF5 and GIF1 expression in *Eucalyptus grandis* tissues. Downloaded from EucGenIE.org. **Top:** *Eucayptus* GIF1 (Eucgr.B02127) **Bottom:** *Eucalyptus* GRF5 (Eucgr.F04420). Units are log2(transcripts per million + 1.

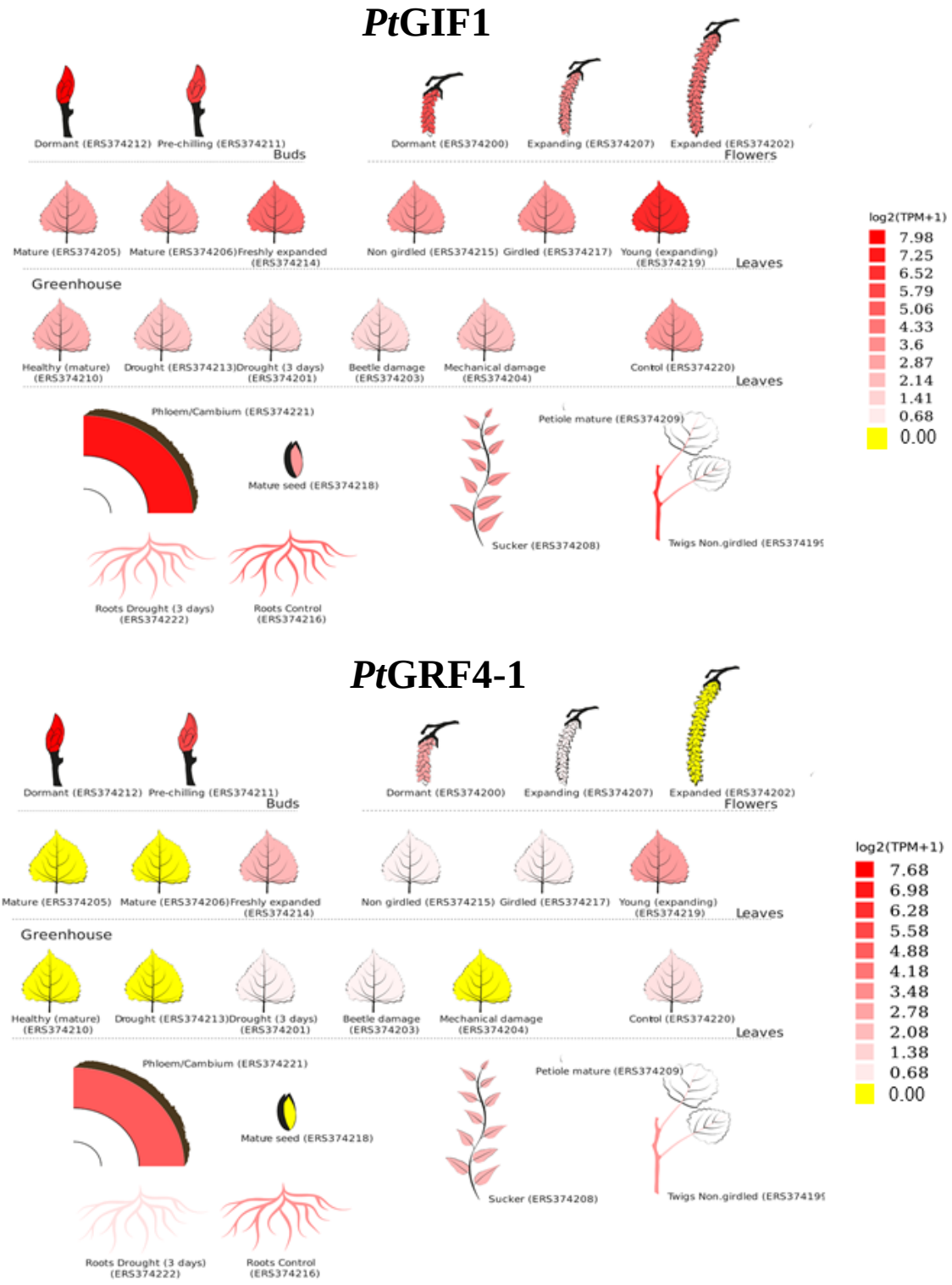

Figure 2.7: Absolute expression heatmap of GRF4-1 and GIF1 expression in *Populus tremula* tissues. Downloaded from PlanGenIE.org. **Top:** GIF1(Potri.019G013100) **Bottom:** GRF4-1 (Potri.013G077500). Units are log2(transcripts per million + 1.

2.3.2 Overexpression of *Citrus* miRNA396-resistant GRF-GIF

2x35S:*Citrus* miRNA396-resistant GRF-GIF overexpression reduced transgenic shoot formation and interacted strongly with genotype in hybrid poplar

In a large (≥15 plates per construct), replicated experiment, 2x35S:*Citrus* miRNA396-insensitive GRF4-GIF1 (rGRF-GIF) significantly reduced the transgenic shoot regeneration rate (plates with ≥1 transgenic shoot) in poplar genotype 353-53 compared with an empty vector control (Figure 2.9) but had little effect on the same metric in 717-1B4. Remarkably, transgenic shoot regeneration frequency of 353-53 with *Citrus* rGRF-GIF overexpression was only 5% of the control vector. This result in 353-53 is significant ($P = 0.03$). In 717-1B4, regeneration frequency with *Citrus* rGRF-GIF overexpression was roughly equal to that of the empty vector control, a difference that was not statistically significant ($P = 0.65$). A Chi-squared test of independence was conducted to explore the effect of 'genotype x transformation construct' interaction on regeneration rate. Genotype and transformation construct are strongly related in terms of regeneration rate ($X^2 = 16.7$; $p = <0.0001$). This relationship is illustrated in Figures 2.8 & 2.9. No other explanatory variables were statistically significant ($P < 0.05$). The *Citrus* rGRF-GIF construct was also tested on a small scale in two eucalypt clones but did not have a statistically significant effect on transgenic callus regeneration ($P < 0.05$) (Appendix B).

In addition to reducing shoot regeneration, *Citrus* rGRF-GIF caused high mortality in shoots that were recovered following regeneration. Five events from each treatment were collected from each genotype and placed in EM 49 days PC (post cocultivation). Of the ten *Citrus* rGRF-GIF events, zero were still living 28 weeks PC, but 80-100% of the empty vector control plants were living. These results are statistically significant ($P < 0.05$) using a Fisher's exact test for both genotypes (717: $P = 0.047$; 353: $P = 0.007$) (Appendix Table A.4).

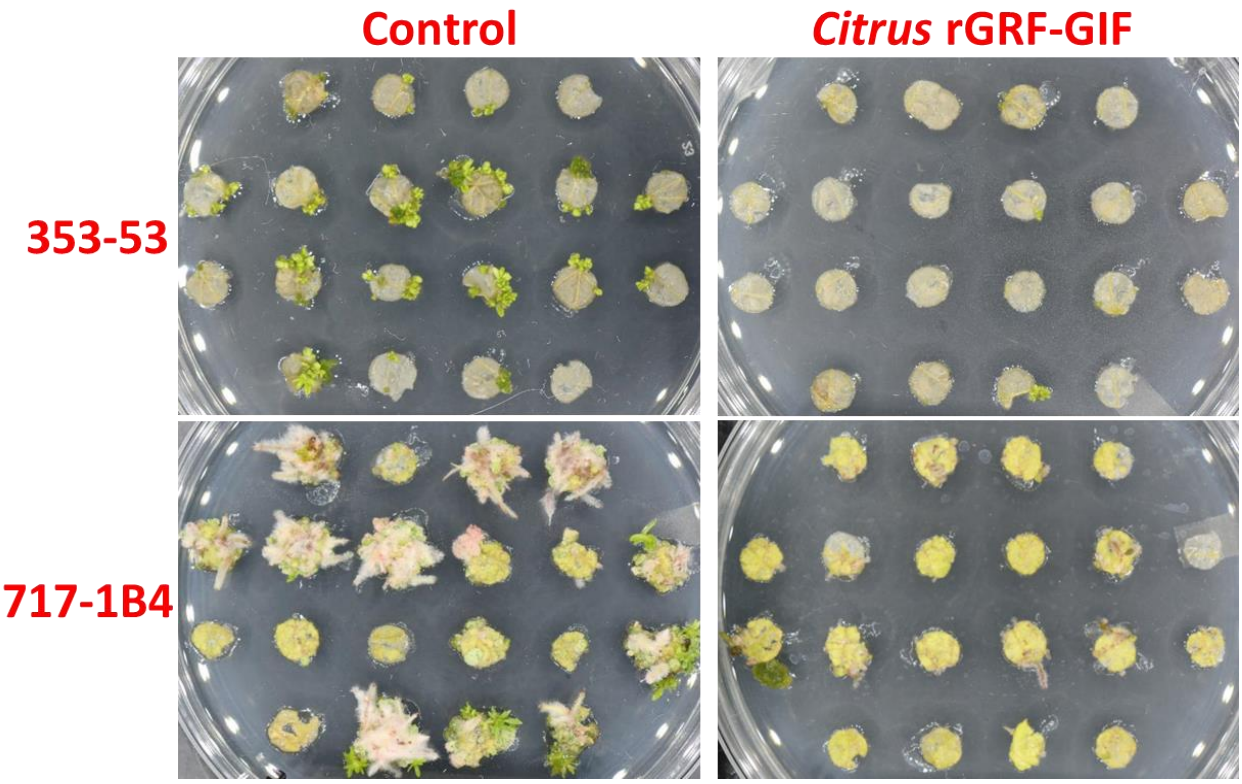

Figure 2.8: Differences in shoot and root formation while overexpressing *Citrus* rGRF-GIF. Whole-plate RGB images showing leaf disk explants from '353-53' (top) and '717-1B4' (bottom) 6-weeks post transformation. Enhanced rooting can be seen for 717-1B4 control in lower-left sector.

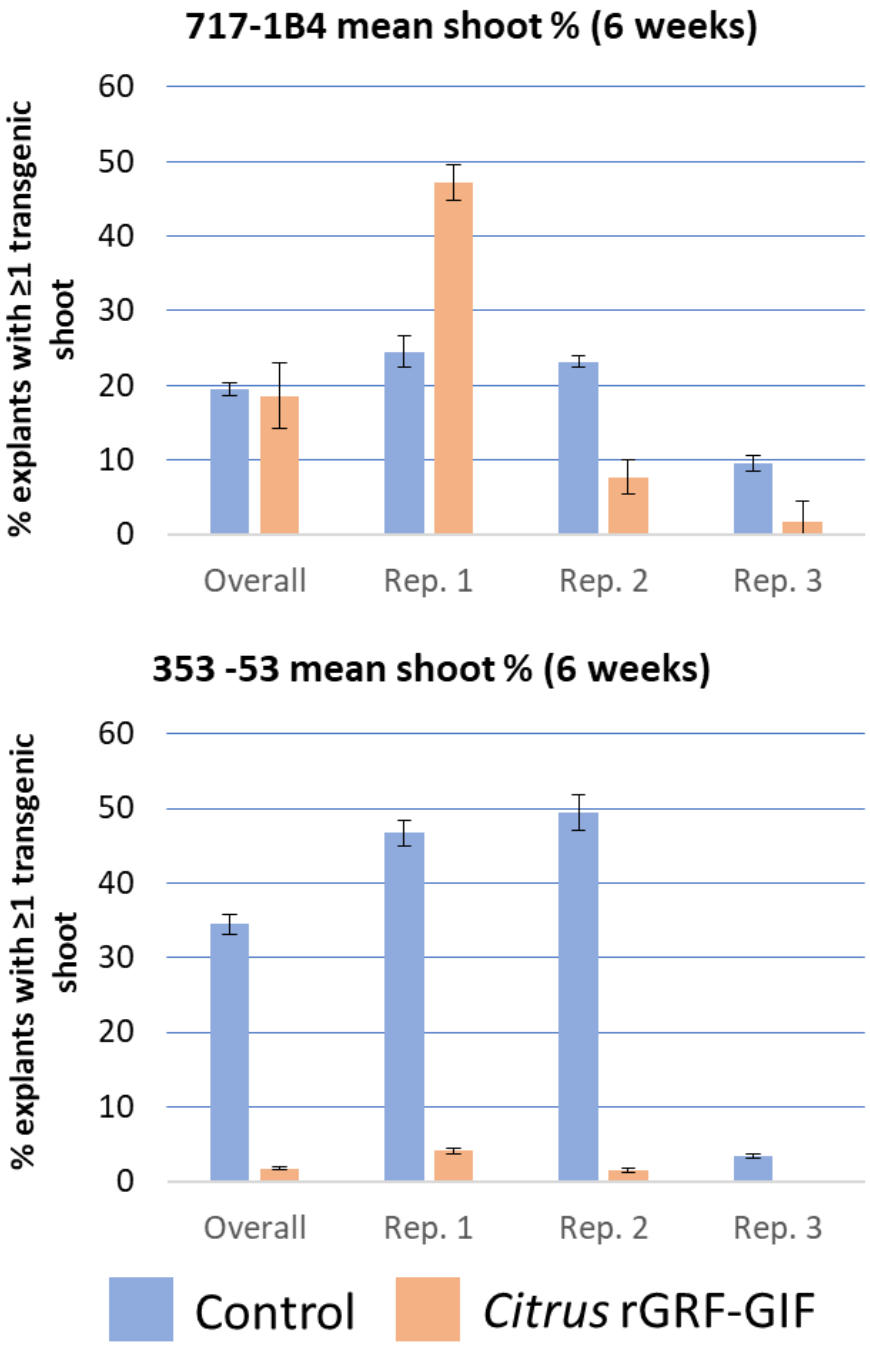

Figure 2.9: Mean percentage of explants with ≥1 transgenic shoot (per plate) 6-weeks post-transformation with Citrus rGRF-GIF. Error bars represent standard error. Rep = replicate. Replicate represents independent experiments transformed at separate times.

2x35S:*Citrus* miRNA396-resistant GRF-GIF overexpression reduced ectopic root formation in 717-1B4 leaf disk explants

When placed on auxin-rich CIM medium, leaf disk explants from 717-1B4 often produced ectopic roots (shown in Figure 2.8, lower left sector). In three separate replicates, leaf disk explants from genotype 717-1B4 transformed with 2x35S:*Citrus* rGRF-GIF had significantly fewer ectopic roots 42 days PT than those transformed with the empty vector control (Figure 2.10). On a plate-wide average basis, the number of explants with ectopic roots in the empty vector control group was 1.84-fold higher than the *Citrus* rGRF-GIF group (68% vs 37%). This result is significant (P = 0.003) using a linear mixed-effects model. No 353-53 explants produced ectopic roots in either the control or *Citrus* rGRF-GIF groups.

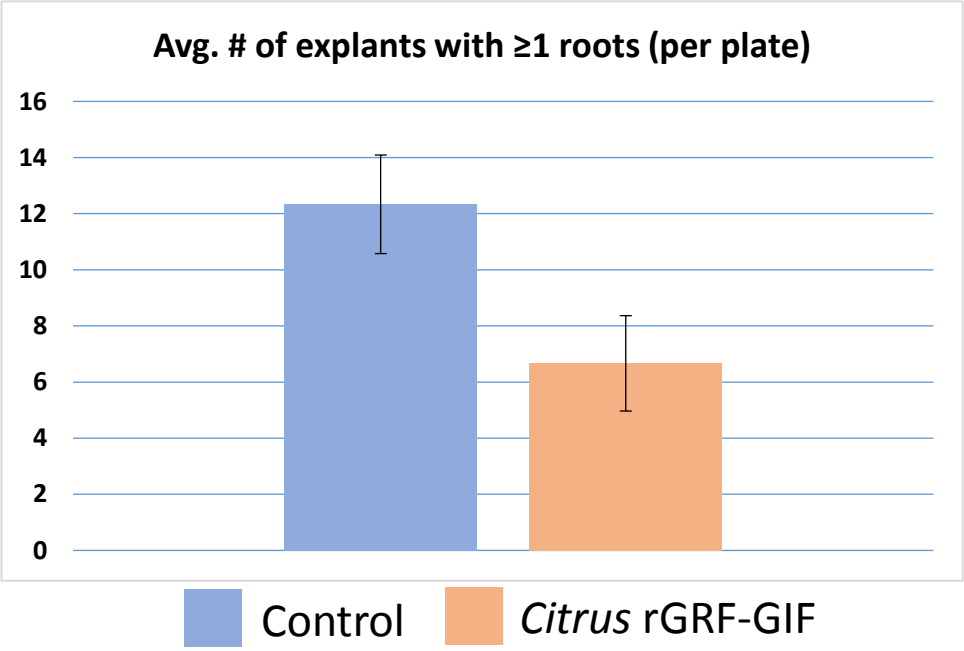

Figure 2.10: Mean number of 717-1B4 leaf disk explants which produced ≥1 ectopic root per plate (n=9 plates/construct).

Genotype	No. independent replicate experiments	Construct	Avg. No. explant/ replicate	Avg. plates/ replicate (20 grid)	Avg. No. explant with transgenic shoot(s)/ plate	Regen. Efficiency (total shoots/ total explants)	% change vs control (P-value) (>|z|)
'717-1B4'	3	Control	91	5	3.6	19.41%	-4.3% (0.65)
		GRF-GIF	108	6	3.3	18.58%	
'353-53'	3	Control	99	6	5.7	34.46%	-95.0% (0.03)
		GRF-GIF	95	5	0.4	1.75%	

Table 2.1: Summary of 2x35S:*Citrus* miRNA396-insensitive GRF-GIF (rGRF-GIF) experiment in two hybrid poplar genotypes. Average number of explants/replicate and plates/replicate were calculated by dividing the total number of plates by the number of replicates (3). Regeneration efficiency is defined as the percent of explants with ≥1 shoot. P-values are calculated using a generalized linear mixed-effects regression model wherein each observation is one plate (20 explants).

2.3.3 Overexpression of *Populus* miRNA-sensitive GRF-GIF

1x35S:*Populus* GRF4-GIF1 enhanced transgenic shoot regeneration in genotype 717-1B4 but not in 353-53

In a large replicated experiment (15 plates per construct), stem explants from hybrid poplar clone 717-1B4 transformed with 1x35S:*Populus* GRF4-GIF1 (*Pt*GRF-GIF) produced transgenic shoots at a higher rate than those transformed with the control vector (19.3% vs 9.3%). However, this result was not significant at the $P < 0.05$ level ($P = 0.067$). Genotype 353-53 produced transgenic shoots at a lower rate than the control vector (7.2% vs 16.0%), however this result is not significant ($P = 0.244$) as the experiments were highly variable (Figures 2.10 & 2.11).

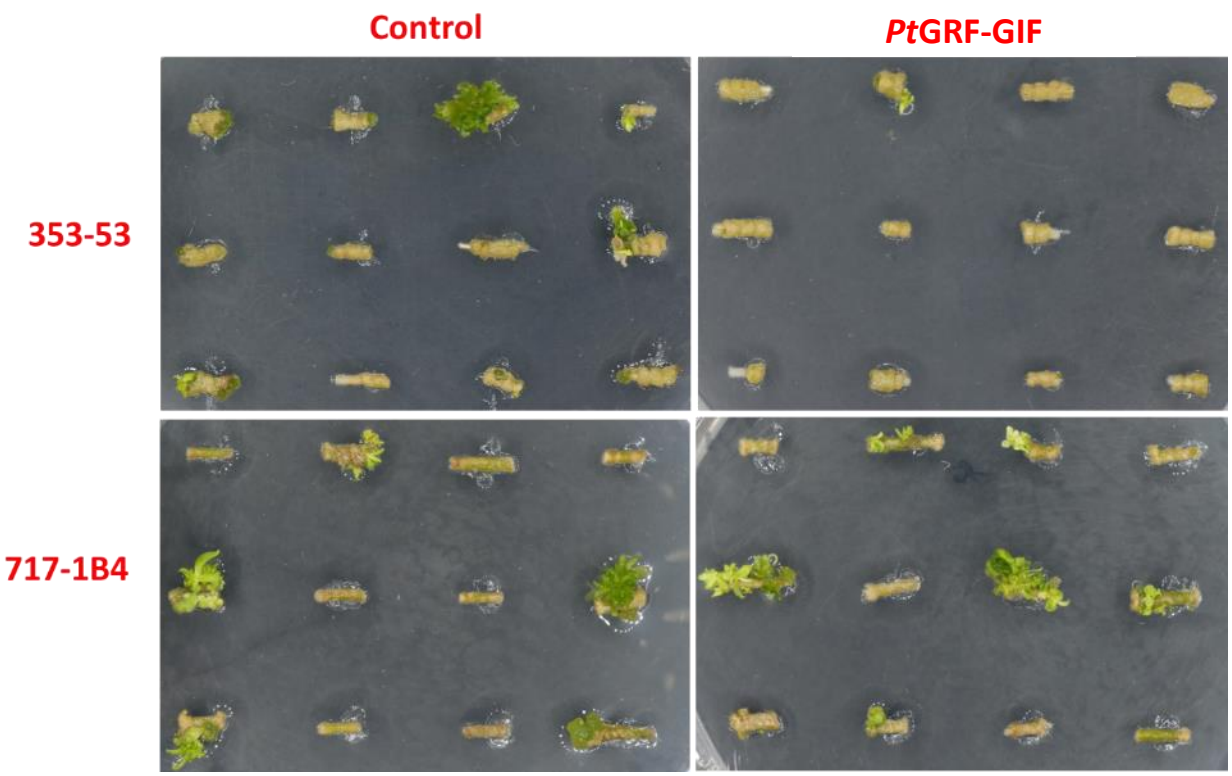

Figure 2.11: Whole-plate images of regeneration in two poplar clones 7-weeks post-transformation. *Pt*GRF-GIF = 1x35S:*Populus trichocarpa* GRF4-GIF1. Images were chosen to be representative of mean regeneration rates.

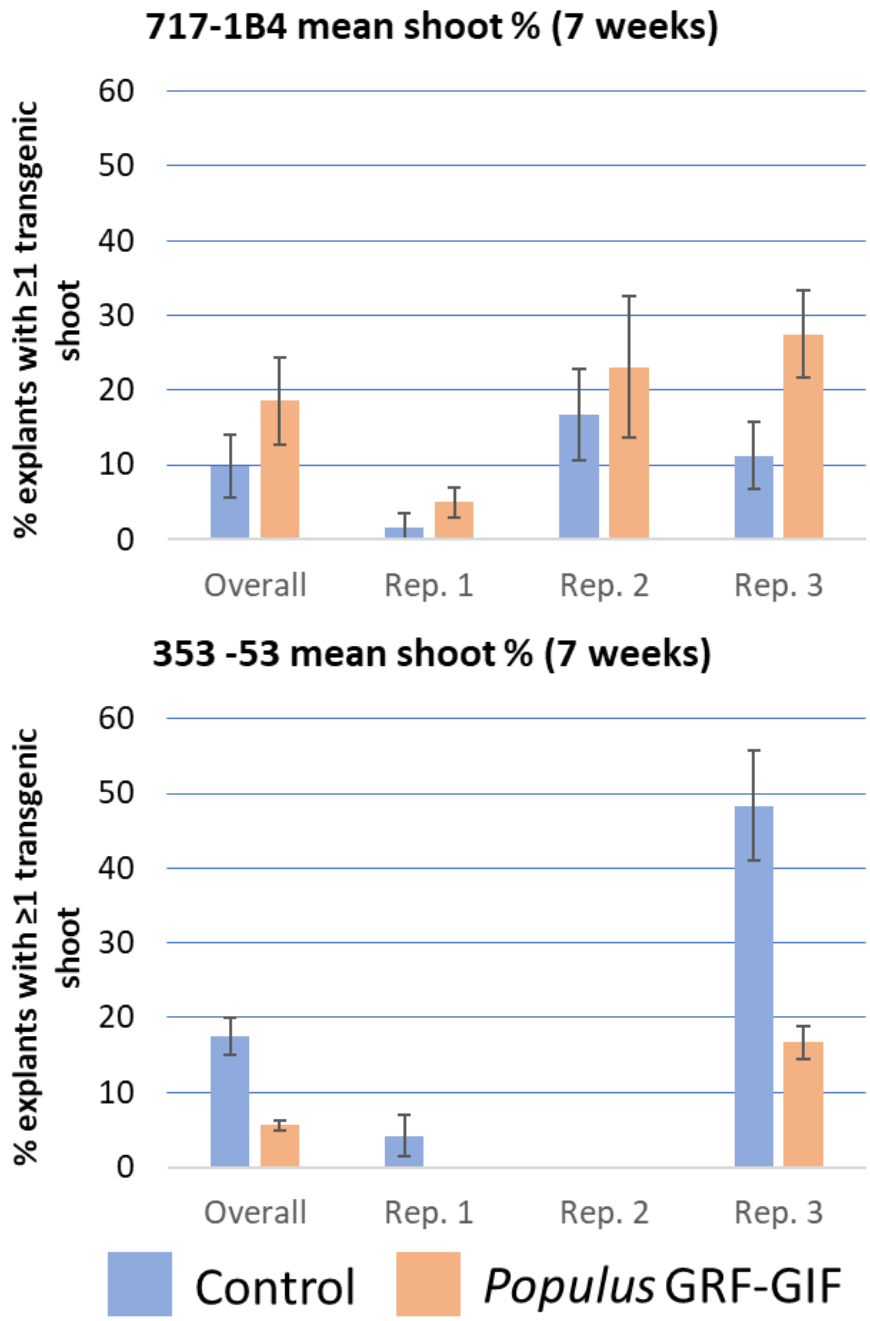

Figure 2.12: Mean percentage of explants with ≥1 transgenic shoot (per plate) 7-weeks post-transformation. Error bars represent standard error. Rep = replicate. 353-53 Rep. 2 had no regeneration in either construct.

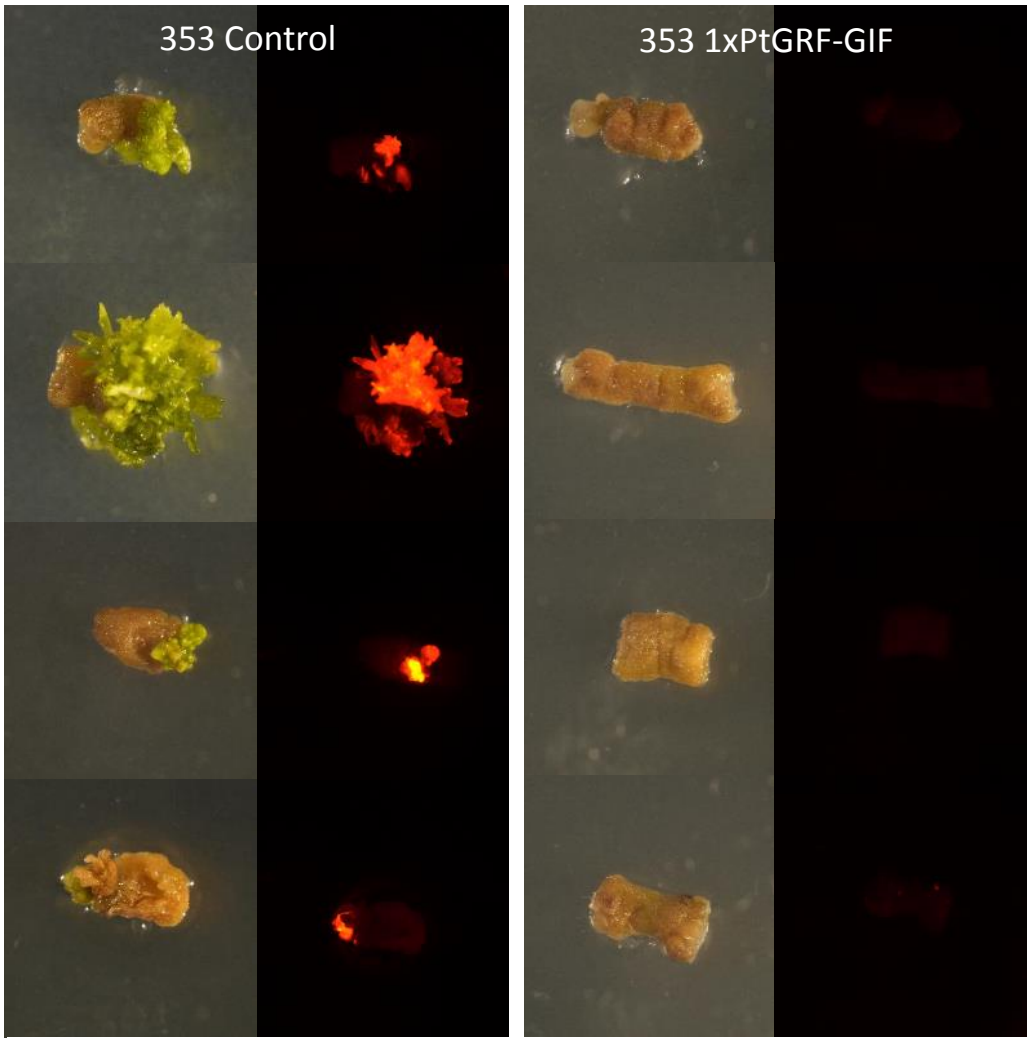

Figure 2.13: Brightfield and epifluorescent images comparing regeneration phenotypes of hybrid poplar 353-53 at 7-weeks post-transformation. Control = empty control vector (left). DsRed reporter is in red. Images were chosen to be representative.

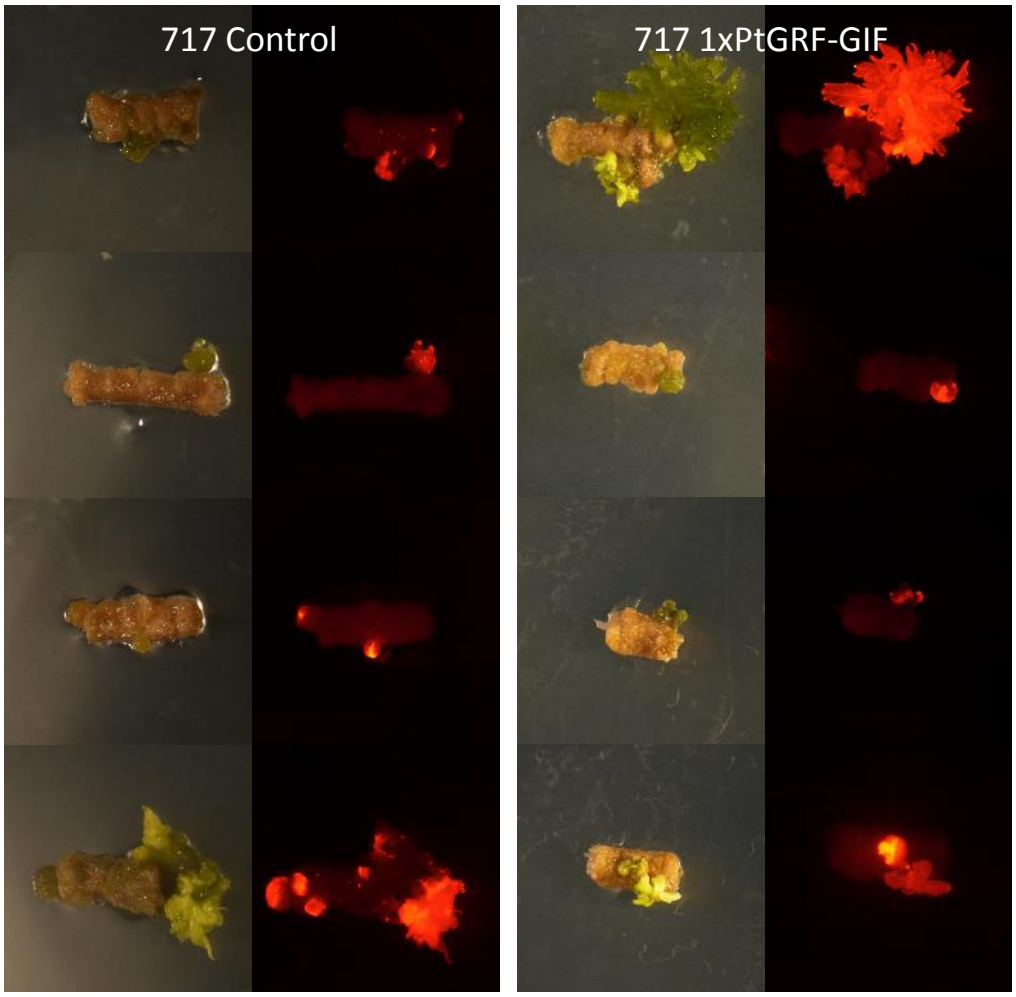

Figure 2.14: Brightfield and epifluorescent images comparing regeneration phenotypes of hybrid poplar 717-1B4 at 7-weeks post-transformation. Control = empty control vector (left). DsRed reporter is in red. Images were chosen to be representative.

1x35S:*Populus* GRF4-GIF1 reduced transgenic callus regeneration

Transgenic callus regeneration, defined as mean number of explants with transgenic callus per plate, was not significantly affected in 717-1B4 even though the percent of explants with transgenic callus decreased from 30% to 18% when comparing *Pt*GRF-GIF to the control (P = 0.38). Transgenic callus regeneration of genotype 353-53 transformed with *Pt*GRF-GIF was 10x lower than those transformed with the control vector. The percent of explants with transgenic callus decreased from 16% in the control group to just 1.6% in the *Pt*GRF-GIF group. This result is significant at the $p < 0.05$ level (P = 0.026). There was a very nearly statistically significant relationship between genotype and transformation construct with respect to mean transgenic callus regeneration (P = 0.053).

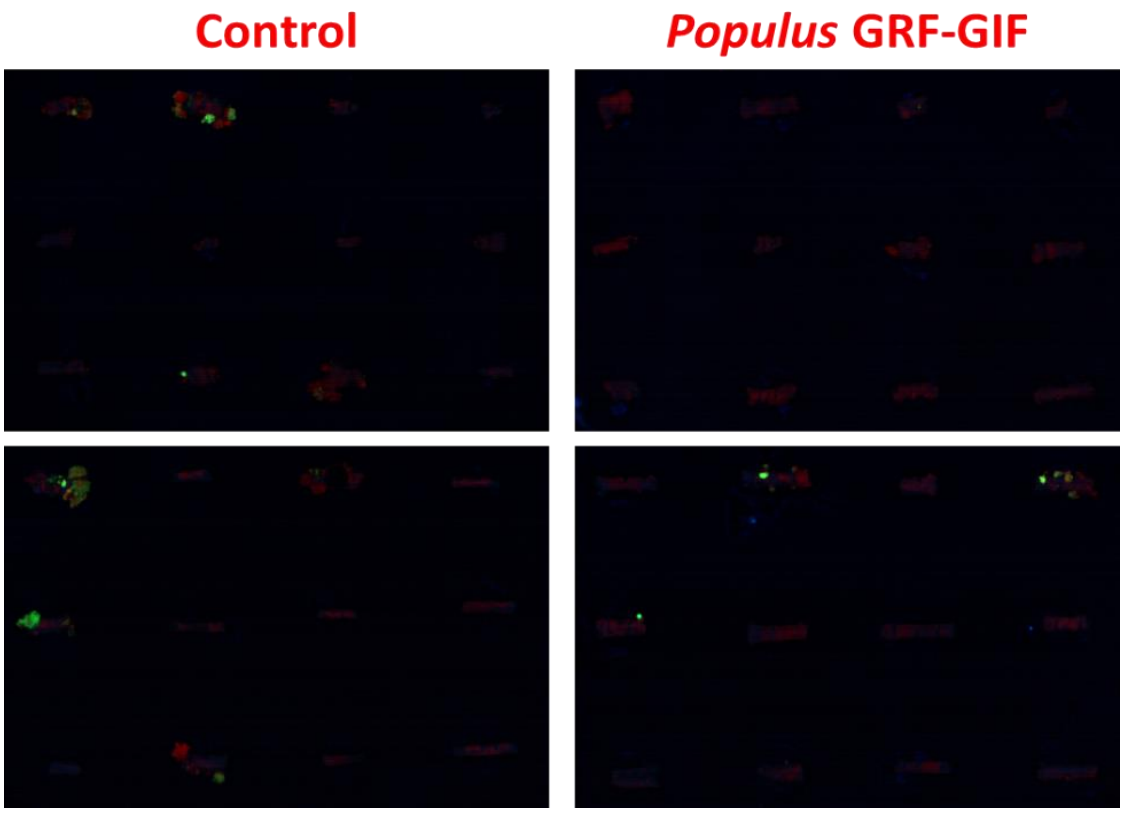

Figure 2.15: Difference in transgenic callus regeneration between two poplar genotypes transformed with *Pt*GRF-GIF. Whole-plate false color fluorescent images showing transgenic callus (green) from '353-53' (top) and '717-1B4' (bottom) stem explants 7-weeks post-transformation. Images were chosen to be representative of transgenic callus means.

Mortality of 1x35S:*Pt*GRF-GIF transformants is highly genotype-dependent

Of the eight 353-53 *Pt*GRF-GIF explants with transgenic shoots that were placed in EM following SIM treatment, all of the shoots died. Shoots derived from the empty vector explants showed no fatality. This result is highly significant using a Fisher's exact test ($P < 0.001$). Curiously, shoots from 717-1B4 explants displayed no mortality. Of the eight *Pt*GRF-GIF explants placed on EM, all maintained healthy shoots (Figure 2.16). There is a significant relationship between mortality and genotype when overexpressing *Pt*GRF-GIF according to a Fisher's exact test ($P = 0.02$) (Appendix Table A.5).

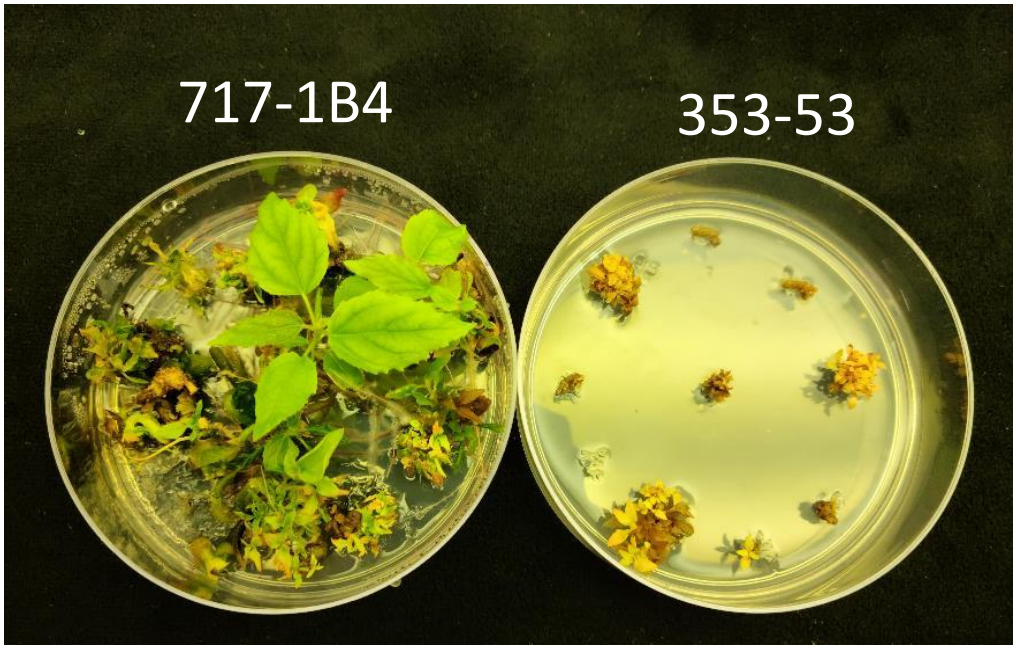

Figure 2.16: Difference in transgenic shoot mortality between two poplar genotypes transformed with *Pt*GRF-GIF. Image was taken 28-weeks post-transformation.

Genotype	No. independent replicate experiments	Construct	Avg. No. explant/ replicate	Avg. plates/ replicate (20 grid)	Avg. No. explant with transgenic shoot(s)/ plate	Regen. Efficiency (total shoots/ total explants)	% change vs control (P-value) (>|z|)
'717-1B4'	3	Control	58	5	1.2	9.8%	+96.9% (0.07)
		GRF-GIF	57	5	2.2	19.3%	
'353-53'	3	Control	69	6	1.8	16.0%	-70.3% (0.24)
		GRF-GIF	74	6	0.8	7.2%	

Table 2.3: Summary of 1x35S:*Populus* miRNA396-sensitive GRF-GIF experiment in two hybrid poplar genotypes. Average number of explants/replicate and plates/replicate were calculated by dividing the total number by the number of replicates (3). Regeneration efficiency is defined as the percent of explants with ≥1 shoot. P-values are calculated using a generalized linear mixed-effects regression model wherein each observation is one plate (12 explants).

2.3.4 Comparison of *Citrus* rGRF-GIF and *Populus* GRF-GIF overexpression

Overexpression of *Citrus* rGRF-GIF (2x35S) and *Populus* GRF-GIF (1x35S) led to slightly different shoot regeneration outcomes (Figure 2.16). Several experimental conditions differed between the two experiments, including the promoter strengths, explant type (stem and leaf vs only stem), and time of regeneration data collection (42 vs 49 days PT). For 353-53, the only variables which had a significant effect on binary shoot regeneration were tissue type ($P = 0.04$) (Figure 2.16) and the *Citrus* rGRF-GIF construct, which differed significantly from the control ($P = 0.02$). For 717-1B4, no significant explanatory variables were found, indicating no significant differences existed between the empty vector control, *Citrus* rGRF-GIF, or *Populus* GRF-GIF.

Figure 2.17: Comparing mean percentages of explants with ≥1 transgenic shoot (per plate) in *Citrus* and *Populus* GRF-GIF experiments. Exp. = experiment, L = leaf explants, S = stem explants. Means are pooled from 3 repetitions. Error bars = standard error.

2.3.5 Additional GRF-GIF constructs tested

In addition to the replicated experiments described so far, I conducted several non-replicated experiments with five more 2x35S:GRF-GIF constructs (Appendix Table A.1), many of which gave statistically significant results based on within-experiment replication. Additional details of those experiments can be found in Appendices C and D. Key findings are summarized below.

2x35S:*Populus* GRF-GIF

- Significantly increased transgenic shoot regeneration in *P. alba* clone '6K10' from 0% in the control construct to 37%. The sample size was 4 plates/construct with an average of 12 explants per plate. There was a statistically significant difference in transgenic shoot regeneration versus the control vector according to a Fisher's exact test (P = 0.01).

2x35S:*Citrus* miRNA-sensitive GRF-GIF

- Reduced transgenic shoot regeneration by 10.6% in 717-1B4 and 30.2% in 353-53 versus the empty vector control construct. There was a statistically significant difference in transgenic shoot regeneration versus the control vector in 353-53 (P = 0.03), but not in 717-1B4 (P = 0.17). Sample size = 6 plates/construct for each genotype (11 explants per plate on average).

2x35S:*Citrus* GRF-GIF with 1 miRNA396 mutation

- Reduced transgenic shoot regeneration by 76.0% in 717-1B4 and 95.3% in 353-53 versus the control construct. There was a statistically significant difference in transgenic shoot regeneration versus the control vector in both genotypes (717-1B4: P < 0.01; 353-53: P = 0.01). Sample size = 7-8 plates/construct for each genotype (11 explants per plate on average).

2x35S:*Vitis* GRF-GIF with 4 miRNA396 mutations

- Reduced transgenic shoot regeneration by 88.7% in 717-1B4 and 51.4% in 353-53 versus the empty vector control construct. There was a statistically significant difference in transgenic shoot regeneration versus the control vector in 717-1B4 ($P < 0.01$), but not in 353-53 ($P = 0.1$). Sample size = 6 plates/construct for each genotype (11 explants per plate on average).

2x35S:*Vitis* GRF-GIF with 4 miRNA396 mutations and glucocorticoid receptor domain for dexamethasone activation

- Poplar: Reduced transgenic shoot regeneration by 54.9% in 717-1B4 and 100% in 353-53 (from 40% to zero). There was a statistically significant difference in transgenic shoot regeneration versus the control vector in 353-53 ($P < 0.01$) but not in 717-1B4 ($P > 0.05$). Sample size = 6-7 plates/construct for each genotype (11 explants per plate on average).
- Eucalypt: Increased transgenic callus formation in SP7 by 31.8% and in Eug21-1 by 23.1%. Transgenic shoot primordia formation increased in SP7 by from 0% to 7.6% and in Eug21-1 from 0% to 10%. None of these results were statistically significant ($P > 0.05$). Sample size = 4-6 plates/construct for each genotype (11 explants per plate on average).

2.3.6 Discussion

Reasons for genotype-dependent results in hybrid poplar

I demonstrated that GRF-GIF overexpression affected transgenic shoot regeneration and shoot survivability in a highly genotype-dependent manner in two hybrid poplar genotypes. While the exact reasons for this are not known, this result is not surprising since plant transformation, even with the use of "DEV" genes, remains highly genotype-dependent in most species (Gordon-Kamm et al., 2019). Additionally, the genus *Populus* has a great amount of genetic diversity within and between species, including for key development genes (Nagle et al., 2022), and this could be one reason for the disparate outcomes observed between genotypes 353-53 and 717-1B4 in this study. Perhaps 353-53 has relatively low miRNA396 expression, which

would increase its sensitivity to GRF overexpression, and 717-1B4 has relatively high miRNA396 expression, which would reduce its sensitivity to GRF. This hypothesis could be readily tested. Alternatively, given the complexity of gene expression in relation to meristem development there may be hundreds of other genetic differences contributing. However, neither this miRNA nor GRF-GIF genes were identified as important to genetic variation of *in vivo* shoot formation in *Populus* by GWAS (Nagle et al. 2022).

Experimental error is a persistent problem in my experiments

Similar to many other studies of *in vitro* regeneration in *Populus* and other genera, I found very high environmental variation in my studies. For example, while I consider the *Citrus* miRNA-resistant and *Populus* miRNA-sensitive GRF-GIF experiments to be rigorous and replicated experiments, the amount of variation between and within the experimental replicates and small sample sizes compromised the reliability of the results. For instance, I saw some evidence that *Pt*GRF-GIF may increase transgenic shoot regeneration in 717-1B4 ($P < 0.1$), but the regeneration of the empty vector control group was consistently lower in these experiments than in the *Citrus* 4-mutation GRF-GIF experiments (Figure 2.16). When these two experiments were compared in one model, no construct was significantly different in 717-1B4 at the $P < 0.1$ level. This reduces my confidence that our results could be replicated. Additionally, I saw large amounts of within-experiment variability, evidenced by frequently large error bars, which means that my estimates for any one experiment are also very imprecise.

The primary way to improve the reliability of regeneration experiments is to reduce the experimental variability. Based on my experiments, it seems safe to assume that the largest source of variability is the amenability to transformation and regenerability of the starting plant material. Averaging this variability across a broader sample of plant material may reduce the experiment-to-experiment variance. For example, instead of using a set number of Magenta boxes (each containing five plantlets) per experimental condition, taking one plantlet from many boxes may reduce the variability of transformation and regeneration. It would be simple to test this hypothesis experimentally. An experiment which uses a single transformation vector, such as the empty vector used in these experiments, to transform material from a few dozen Magenta boxes from multiple genotypes would give us an idea of how much variability exists between boxes for a given genotype. The amount of variability from these experiments could be used to

determine how many boxes would need to be used in routine transformation experiments to reach an acceptable error threshold. Taking this idea even further, perhaps individual plantlets within each Magenta box could given a unique identifier and randomized so that each experiment uses a random sample of the larger plantlet population.

Another way to reduce variability would be to substantially increase sample sizes. However, there are legitimate logistical considerations which limit the practical maximum sample size. The largest constraint on practical sample size is the student/technician labor. Transformation and subsequent subculturing can take significant portions of the workday. This limits sample sizes, especially if multiple experiments are ongoing simultaneously.

Improving the efficiency of experiments (defined as technician time/experiment) could free up time to handle additional sample size. Techniques which automate transformation, subculturing, and/or scoring of plant material may improve efficiency and reduce variability. Using mechanical means to dismember and wound plant material during transformation rather than manual means could reduce the variability of wounding depth/amount and speed up the process. Subculturing entire plates in one movement rather than subculturing each explant individually and automating the scoring of explants could also free up significant portions of technician/student time and allow for larger sample sizes in experiments.

Our lab has taken significant strides toward adopting these efficiency 'boosters.' Using auxotrophic *Agrobacterium* strains eliminates the need for washing, saving ~2 hours per replicate. Recently, we have begun using a common kitchen blender in the transformation process, saving ~2-3 hours per experiment and subculturing entire plates at once by placing explants on a sterile filter paper sheet on top of the medium, which may save a technician an additional 2-3 hours per experiment. We have also begun using a high-throughput automated phenomics pipeline to assess regeneration, which can save 1-2 hours per replicate. All of these efficiency 'boosters' added together save 5-10 hours per replicate. If a typical replicate takes approximately 12 hours of active time, the sample size could be easily doubled using all of these 'boosters' together.

In summary, we could reduce experimental variability by adopting the following practices (ranked by order of perceived impact):

1. Combining plantlets from many Magenta boxes, if this is indeed found to be an important source of variation
2. Increasing the practical sample size via gains in efficiency of sample processing
3. Using mechanical means to wound explants, if indeed this gives comparable or larger rates of regeneration or transformation, and can be used in our automated phenotyping system

GRF-GIF may reduce shoot induction in poplar by interfering with pluripotent callus formation

My results show that overexpression of miRNA-resistant and miRNA-sensitive GRF-GIF chimeras either failed to significantly improve, or acutely repressed, transgenic shoot regeneration. Impairment was especially striking for genotype '353-53.' I also showed that GRF-GIF overexpression led to decreases in callus and ectopic root formation. This suggests a repression of the callus/lateral root meristem (LRM) cell fate (Atta et al., 2009) . Both *PLETHORA1* (*PLT1*) and *SCARECROW* (*SCR*) transcription factors play important roles in callus formation, which closely mimics the formation of LRMs (Sugimoto et al., 2019). *SCR* is expressed in the quiescent center (QC) of the root meristem, where it regulates stem cell identity, in part by inducing the expression of *WUSCHEL-RELATED HOMEOBOX5* (*WOX5*), the master regulator of root meristem identity (Sarkar et al., 2007). It is also known that *Arabidopsis AN3/GIF1,* perhaps as a heterodimer with one or more GRFs, is involved in the regulation of *PLT1* and *SCR* transcription factors, and that the loss-of-function *an3/gif1* mutants have enlarged root meristems and increased *PLT1* expression (Ercoli et al., 2018).

In *Arabidopsis*, GRFs are excluded from the stem-cell niche of the root meristem by miRNA396 expression, but they are highly upregulated in the daughter cells of stem cells, the transit-amplifying cells (TACs). In the TACs, GRFs promote rapid division and organogenesis, and loss-of-function *grf* mutants have larger root apical meristems but reduced root growth. In miRNA396-resistant GRF experiments in *Arabidopsis*, GRFs induced the formation of a distorted QC, which compromised the ability of the root meristem to continue stem cell division (Rodriguez et al., 2015). If GRF4-GIF1 overexpression decreases the transcription of *PLT1* and *SCR*, leading to a disordered root meristem, this could explain the decrease in both callus and ectopic root formation that I observed.

I reported a decrease in callus in a miRNA-sensitive (wild-type) GRF4-GIF1 overexpression experiment, which should be post-transcriptionally controlled by miRNA396. However, *PLT1* expression is required for miRNA396 expression in the root meristem (Rodriguez et al., 2015). Additionally, GRFs participate in an auto-activation of their own transcription (Kim, 2019b). Thus, overexpressing GRF4-GIF1 with a strong constitutive promoter (such as CaMV35S) may lead to reductions in miRNA396 expression, creating a 'vicious cycle' wherein repressed *PLT1* expression results in repressed miRNA396 expression, which in turn allows more GRF4-GIF1 protein to be translated. This increase in GRF4-GIF1 may increase transcription of native GRFs, which further repress *PLT1*, and then the cycle repeats. This may ultimately lead to a disordered root meristem and/or a reduction in callus stem cells which are necessary for an indirect *de novo* shoot organogenesis process.

The acquisition of pluripotency in callus is a critical step in DNSO, as exemplified by the low rates of shoot regeneration in *plt1* and *scr Arabidopsis* mutants (Kim et al., 2018). Following pluripotency acquisition, a subpopulation of pluripotent callus cells expressing transcription factors associated with the root stem cell niche acquire shoot identity upon introduction to cytokinin during the shoot-induction phase (Liu et al., 2018). Thus, the formation of pluripotent callus cells expressing *PLT1* and *SCR* is a critical step in DNSO. Poplar transformation relies on a long callus-induction phase (21d), and another 21-28d of shoot-induction is required to induce calli to form shoots. Perhaps the overexpression of GRF-GIF in my study reduced the formation of pluripotent callus, which later limited the number of cells competent for shoot identity acquisition.

GRF-GIF may not promote DNSO in mature tissues

To my knowledge, none of the published reports of GRF-GIF overexpression have used vegetatively propagated explant tissues. The reports in wheat (*Triticum spp.*), rice (*Oryza sativa*), citrange (*Citrus x insitorum*) (Debernardi et al., 2020b), hemp (*Cannabis sativa*) (X. Zhang et al., 2021), and watermelon (*Citrullus lanatus*) (Feng et al., 2021) have all used either embryonic or seedling-derived explant tissues. It is possible that the positive effect on transgenic shoot regeneration may be limited to juvenile tissues such as embryos, cotyledons, hypocotyls, and epicotyls. It is well-known that juvenility of the explant tissue is an important factor for susceptibility of explants to *Agrobacterium* and for subsequent cell division and regeneration (Chakraborty et al., 2020; Davis et al., 1981; Han and Han, 2016). Testing GRF-GIF overexpression in *Populus* hypocotyls alongside clonally-propagated material could elucidate whether this is true. However, it would be highly preferrable to use clonally-derived explants rather than seedling-derived explants in clonally-propagated plants such as *Populus.* Perhaps using floral-derived explants such as stamens, pollen, or sepals would have better success, as these tissues have been used in other recalcitrant species with some successes (Kamaté et al., 2000).

Summary

The effect of GRF-GIF in different poplar genotypes is clearly highly variable, and highly genotype and GRF-GIF gene dependent. Clearly, they do not indicate a consistent benefit for transgenic shoot regeneration. However, scope of inference with respect to the value of GRF-GIF in Populus is limited by the narrow sample of genotypes and genes studied, limited experimental replications (maximum of two), very high within and between experiment variation, and low sample sizes given the extent of variation observed. Due to the often-disparate regeneration outcomes in the two genotypes tested, a much wider set of *Populus* genotypes is clearly needed to make any generalizable inferences about the effect of GRF-GIF overexpression in *Populus.* In addition, my work has only scratched the surface about the possible genes (GRF and combinations with others), promoters, and expression control systems that might be useful.

Due to the interplay between GRF and GIF and the root meristem/callus development pathway, an expression analysis of key genes involved in this pathway using RT-qPCR may help

us to better understand why GRF-GIF did not promote *de novo* shoot organogenesis in my study. Widening this study to include key shoot meristem regulators, such as *SHOOT MERISTEMLESS, WUSCHEL,* and others, may uncover even more clues as to what really happens during GRF-GIF overexpression. This knowledge may help identify where in the regeneration process GRF-GIF overexpression may promote shoot organogenesis in *Populus.*

Chapter 3: Conclusions

Summary of significant findings from this project:

P-values were calculated using a binomial generalized linear mixed-effects model (unless otherwise noted) comparing the GRF-GIF construct to an empty vector control. Each construct vs, control x genotype was replicated (in time) three times.

Citrus rGRF-GIF

- Interacted strongly with genotype (Chi-squared: $P = 0.0004$)
- Reduced prevalence of transgenic shoots in genotype 353-53 versus an empty vector control ($P = 0.03$)
- Reduced prevalence of ectopic root formation on leaf disk explants from genotype 717-1B4 versus an empty vector control (linear mixed-effects model: $P < 0.003$)
- Increased mortality of transgenic shoots placed in elongation medium versus an empty vector control (Fisher's exact test: 717-1B4: $P = 0.047$; 353-53: $P < 0.001$)

Populus GRF-GIF

- Increased prevalence of transgenic shoots in 717-1B4 versus an empty vector, although not statistically significant ($P = 0.07$)
- Reduced prevalence of transgenic calli in genotype 353-53 versus an empty vector control ($P = 0.03$)
- Interacted strongly with genotype in terms of shoot mortality on elongation medium (Fisher's exact test: $P = 0.02$)

Key takeaways from GRF-GIF experiments in hybrid poplar

In this study, I found that GRF-GIF overexpression had a variable effect on transgenic shoot regeneration. Additionally, I found that GRF-GIF overexpression interacted strongly with genotype in two regenerable hybrid poplar genotypes with regard to both transgenic shoot regeneration and shoot survivability. Much more optimization testing will be necessary before GRF-GIF can be considered a useful tool in poplar transformation. Based on my results, it seems likely that different genotypes will respond very differently to different conditions (e.g., promoter strength, GRF-GIF source, miRNA-sensitivity level). I also showed that constitutive GRF-GIF overexpression negatively impacts transgenic callus and root regeneration, which may ultimately limit its usefulness in an indirect transformation system such as ours.

Areas of future research

Exploring alternative promoters: I found that using a single CaMV 35S promoter to drive the native *Populus* GRF4-GIF1 chimera promoted shoot regeneration in one hybrid poplar genotype while the double CaMV 35S promoter driving a closely related GRF4-GIF1 chimera from *Citrus* had almost no effect on shoot regeneration. Further exploration of weaker constitutive promoters, including the nopaline synthase promoter from Agrobacterium, may result in enhanced shoot regeneration. Additionally, using plant developmental promoters, such as the *STM* promoter, may confine GRF-GIF expression to a more optimal temporal and spatial window for promotion of meristem development. Although previous studies in our lab have found inducible expression systems (e.g., dexamethasone and/or estradiol) to be leaky, they may be useful in determining the approximate developmental window in which GRF-GIF promotes transgenic shoot regeneration. With this knowledge, a developmental promoter with a narrow expression window may be selected to drive GRF-GIF.

Gene excision: The hypothesis presented in the discussion posits that GRF-GIF generally harms *de novo* organogenesis (especially callus development) beyond a narrow developmental window. Gene excision is one way to 'confine' GRF-GIF expression to a narrow temporal range. Our lab has been working towards a functional gene excision system, including *Cre* recombinase-mediated excision and CRISPR/Cas excision (Goralogia et al., 2021b). If an efficient excision system comes from this work, GRF-GIF should be studied again, perhaps using data from inducible activation experiments to determine the optimal time to excise GRF-GIF.

CRISPR-activation: My research showed that overexpressing the native GRF-GIF from poplar with a 35S promoter enhanced shoot regeneration in one poplar genotype. A new technique used to overexpress native genes uses a dead Cas9 protein (dCas9) fused to an activation domain to promote transcription of the native gene of interest (Cheng et al., 2013). Using dCas to promote transcription of GRF4 and GIF1 may promote shoot regeneration, and could also be coupled with an inducible activation system (e.g., dexamethasone) or excision to confine GRF-GIF upregulation to a specific temporal window.

Altruistic (non-integrating) transformation: Constitutive GRF-GIF expression appeared to frequently impair transgenic shoot regeneration in poplar, but perhaps an altruistic approach would avoid the negative effect on shoot regeneration while still reaping the early organogenic benefit of GRF-GIF expression. In an altruistic transformation, *Agrobacterium* containing different expression vectors would be mixed together. Some would carry only a selectable marker (fluorescent protein and/or antibiotic resistance enzyme) while others would carry a GRF-GIF gene. The T-DNA harboring the GRF-GIF gene coding sequence would be transiently expressed in a fraction of the cells which have stably integrated the marker T-DNA. Once the GRF-GIF protein is 'turned over,' the cell could then regenerate normally. This approach has been highly successful in monocots using other DEV genes that necessitate excision for regeneration of healthy plants (Hoerster et al., 2020).

In combination with other DEV genes: While the exact mechanism by which GRF-GIF enhances regeneration is not clear (Debernardi et al., 2020b; Feng et al., 2021; Kong et al., 2020; Luo & Palmgren, 2021; Zhang et al., 2021), it is my hypothesis that the GRF-GIF complex enhances cell proliferation in a wide range of cell types, including proto-meristematic zones. In

the past, combinations of DEV genes have been used with successful outcomes, especially the *BABY-BOOM* and *WUSCHEL* (*WUS*) combination in monocots (Gordon-Kamm et al., 2019; Lowe et al., 2016). Perhaps the effect of GRF-GIF could be enhanced through the addition of other DEV genes such as *WUS*, which defines meristematic zones. As mentioned previously, GRF-GIF may repress *PLETHORA1* expression, which could in turn have unintended consequences for callus development. Overexpressing *PLETHORA1* and GRF-GIF may overcome this.

Another potential DEV gene, *CUP-SHAPED COTYLEDON 1* (*CUC1*), is known to increase shoot meristem proliferation in *Arabidopsis* calli (Daimon et al., 2003). If the primary mechanism of GRF-GIF is encouraging cell division and growth, it may synergistically enhance the effect of *CUC1*. Recently, a report showed that *PLETHORA5* (*PLT5*), a regulator of *WUS* expression in meristems, could promote *de novo* shoot formation *in planta* and *in vitro* in a wide range of plant species (Lian et al., 2022). Since *PLT5* is upstream of *WUS*, overexpressing it in combination with GRF-GIF may simultaneously increase shoot meristem identity in calli and enhance the growth rate of the new meristems.

There are many more combinations of genes which may enhance the effect of GRF-GIF, but the interactions of such genes will be difficult to predict. It is very likely that each gene would require a different temporal range, thus constitutively expressing multiple DEV genes is likely not a practical solution to overcome recalcitrance. Using two or more induction systems (for two or more DEV genes) or a combination of excision and induction would allow for more precise timing of DEV gene expression. An experiment which focuses on the optimal expression level and timing of each gene and combinatorial effects of genes would take many years to accomplish using one-factor experiments. A novel methodology for testing multiple *in vitro* factors (e.g., DEV genes, hormones, phytonutrients, etc.) and their interactions simultaneously is "Design of Experiment" (DoE). DoE uses geometric modeling to determine which combinations of factors are worth testing and which are not, thus reducing the overall time and effort required to test multiple conditions and their interactions (Niedz & Evens, 2016). DoE has been proposed as a novel methodology for testing tissue culture conditions, and DEV genes could be viewed as a tissue culture condition which interacts with medium, genotype, and potentially other DEV genes to promote, repress, or leave unchanged the rate of regeneration.

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APPENDICIES

Appendix A: Main text supplementary information.

- R code and associate data are available upon request.

Table A.1 (below): Summary of all experiments detailed in this thesis. Number of mutations (e.g., 4-mut) indicates number of miRNA396 binding-site mutations (in bp). WT = no mutations. Independent experimental replicate represents a set of plates (control and GRF-GIF) transformed at separate times. Different genotypes were kept on separate plates, but the number of plates shown here is pooled. Percent change vs control represents the % and direction of change versus the empty vector control. For poplar, this change refers to transgenic shoot regeneration. For eucalypt, this number refers to transgenic callus regeneration. P-values represent comparison of transgenic shoot or callus regeneration compared with an empty vector control construct. P-values for experiments with 3 experimental replicates are calculated from pooled means using a binomial GLMM. P-values for non-replicated experiments are calculated using Kruskal-Wallis non-parametric ANOVA and a pairwise Wilcoxon rank sum test with corrections for multiple comparisons.

Table A.1: Summary of all experiments detailed in this thesis.

Experiment ID	Species transformed	Construct	Number of independent experimental replicates	Total number of plates (explants)	Number of explants per plate	Date range for experiments	Location of Data in thesis	% change vs control (P-value)
E1	Poplar	2x35S:*Citrus* 4-mut GRF-GIF	3	22 (411) 18 (326) 22 (402)	20	1/2021-4/2021	Main text	717: -4.3% (0.65) 353: -95.0% (0.03)
E2	Poplar	1x35S:*Populus* GRF-GIF	3	22 (272) 19 (234) 22 (266)	12 12 12	11/2021-4/2022	Main text	717: +96.9% (0.07) 353: -70.3% (0.24)
E3	Poplar	2x35S:*Citrus* GRF-GIF	1	24 (290)	12	4/2021-5/2021	Appendix	717: -10.6% (0.17) 353: -30.2% (0.03)
E4	Poplar	2x35S:*Citrus* 1-mut GRF-GIF	1	31 (375)	12	4/2021-6/2021	Appendix	717: -76.0% (<0.01) 353: -95.3% (0.01)
E5	Poplar	2x35S:*Vitis* 4-mut GRF-GIF	1	24 (282)	12	4/2021-6/2021	Appendix	717: -88.7% (<0.01) 353: -51.4% (0.1)
E6	Poplar	2x35S:*Vitis* 4-mut GRF-GIF (+DEX)	1	43 (475)	12	1/2022-3/2022	Appendix	717: -54.9% (0.46) 353: -100% (<0.01)
E7	Poplar	2x35S:*Populus* GRF-GIF	1	8 (81)	12	9/2021-2/2022	Appendix	6K10: N/A (control had no regeneration)
E8	Eucalypt	2x35S:*Citrus* 4-mut GRF-GIF	3	4 (45) 4 (45) 8 (92)	12	1/2021-4/2021	Appendix	SP7: +12.5% (0.93) Eug21-1: +57.1% (0.12)
E9	Eucalypt	2x35S:*Vitis* 4-mut GRF-GIF (+DEX)	1	19 (206)	12	1/2022-3/2022	Appendix	SP7: +31.8% (0.11) Eug21-1: +23.1% (0.16)
E10	Eucalypt	1x35S:*Eucalyptus* GRF-GIF	1	8 (96)	12	1/2022-3/2022	Appendix	N/A

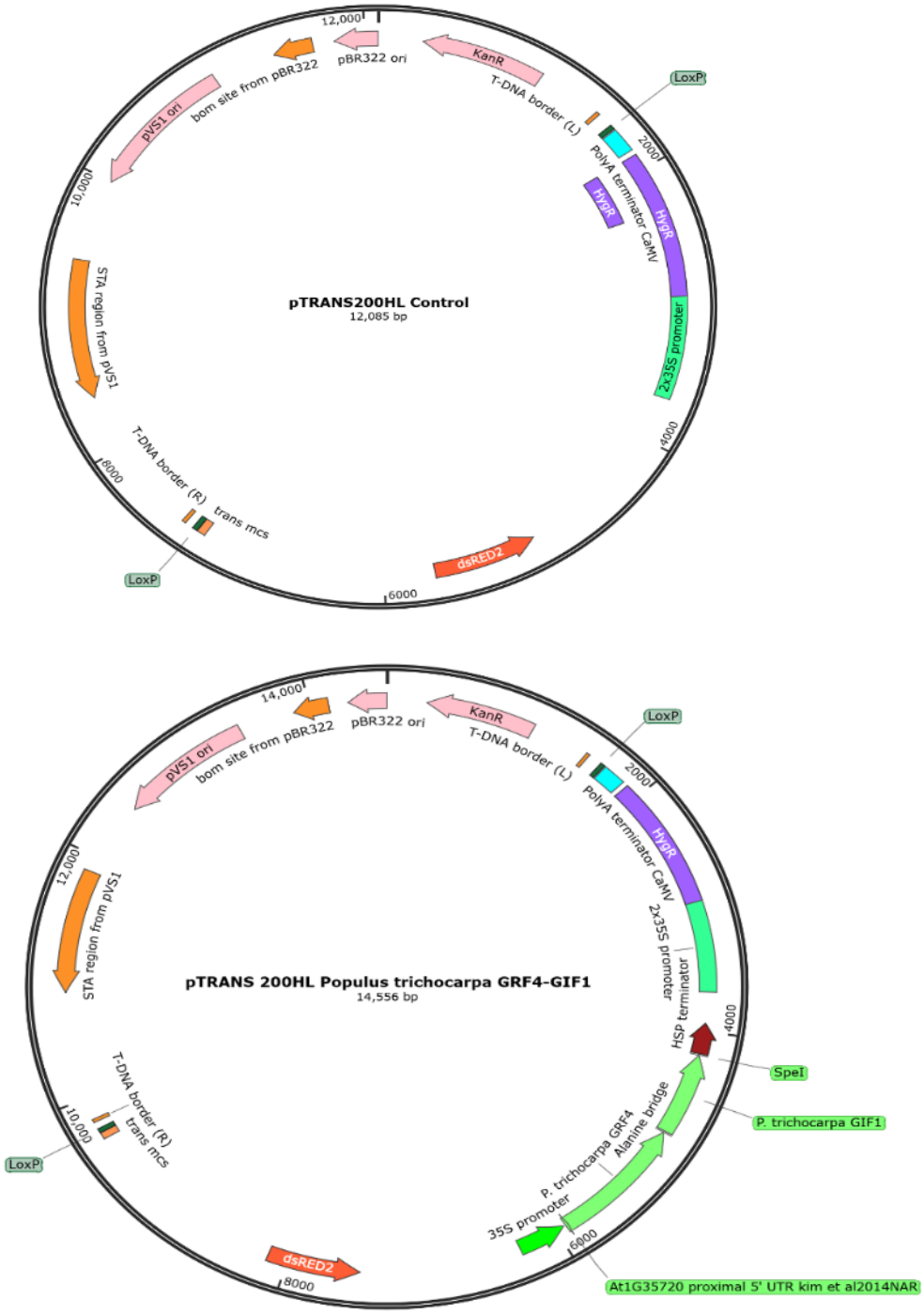


Figure A.1: Detailed plasmid maps of the binary plasmids used in this study. The same control vector (top) was used in all experiments. *Populus* GRF-GIF map is shown here (bottom). All GRF-GIF plasmids used were identical in layout.

E. grandis GRF5-GIF1 construct

The *E. grandis* GRF5-GIF1 construct shown here was used in a non-replicated experiment in genotype 'Eug21-1,' however the experiment empty vector group had no transformation, presumably from nonviable *Agrobacterium.* Data from this experiment is summarized in Figure A.3

>E.grandis|GRF5-GIF1

atgagtgggagtggaagtggaagaatgatgaacaggtattatccattcacagaggcacagtggcaagagctagagacacaagcc
cttgtctacaaatacatggtgtcaggactccaagtcccatctgagctcatcttcagcgttagaaggagcctggagtccaactcctcct
cactctcctttggcccattccctcctcatcaggtgggtgggtggggtgagttccaggcgggttatggcaggaaagcagaccctgag
ccagggaggtgcagaagaacagatgggaagaaatggaggtgctccaaggaggcctaccctgactccaagtactgcgagaagca
catgcaccgaggcaagcaccgttcaagaaagcatgtggaacaacagcaacagccgccagccttgtcgatcggcgccgccgcttg
tccaccgacttcttcatgcattatctcggctagcggtgaacacgaataccaccacccaacactccatcccttcatgactactacttcttc
ctcctcctccgctaggcctgctccagatgcgggtccattccctcatccgtatgaagcaagcagccaccaccttcagcacaactacgt
gctgaattcttattctcagaaccataaagatggcagggcggcggccgccatgcagcagcacctgatgcagatgcagcccatgatg
gccgcttattatccgaacaacgtcaccaccgatcacattcaacagtatttggacgagaacaagtcactgattttgaagatcgttgaga
gccaaaactgtgggaagctgagcgaatgtgcagagaaccaggctaagctacagagaaacctcatgtaccttgctgctatcgctga
ttctcaacctcagccaccgactttgcattctcagtttcccaccagtaccattgcacagcctggatcgtactatgtgcagcaccagcaa
gctcagcagatgaccccacagtccctcatggctgctcgatcttccatgctgtacacccagcaacaaccctatccctcattgcacggc
cagctaagcatgagctccggctcagggctgaacattttgcaaagcgagagcagccactcaggaggtagtggggccctcggtggc
ggaggctttcctgagggcttgcaggttggtgggaggggaatggcgggtggaaacaagcatgatgttgggagtagcagctcggg
cgaaggacgaggtggcggccagggtggagcagacggcggcgagaccctctacttga

Figure A.2: Sequence of *E. grandis* GRF5-GIF1 chimera produced in this study. Gray = *E. grandis* GRF5, green = alanine bridge linker, blue = *E. grandis* GIF1.

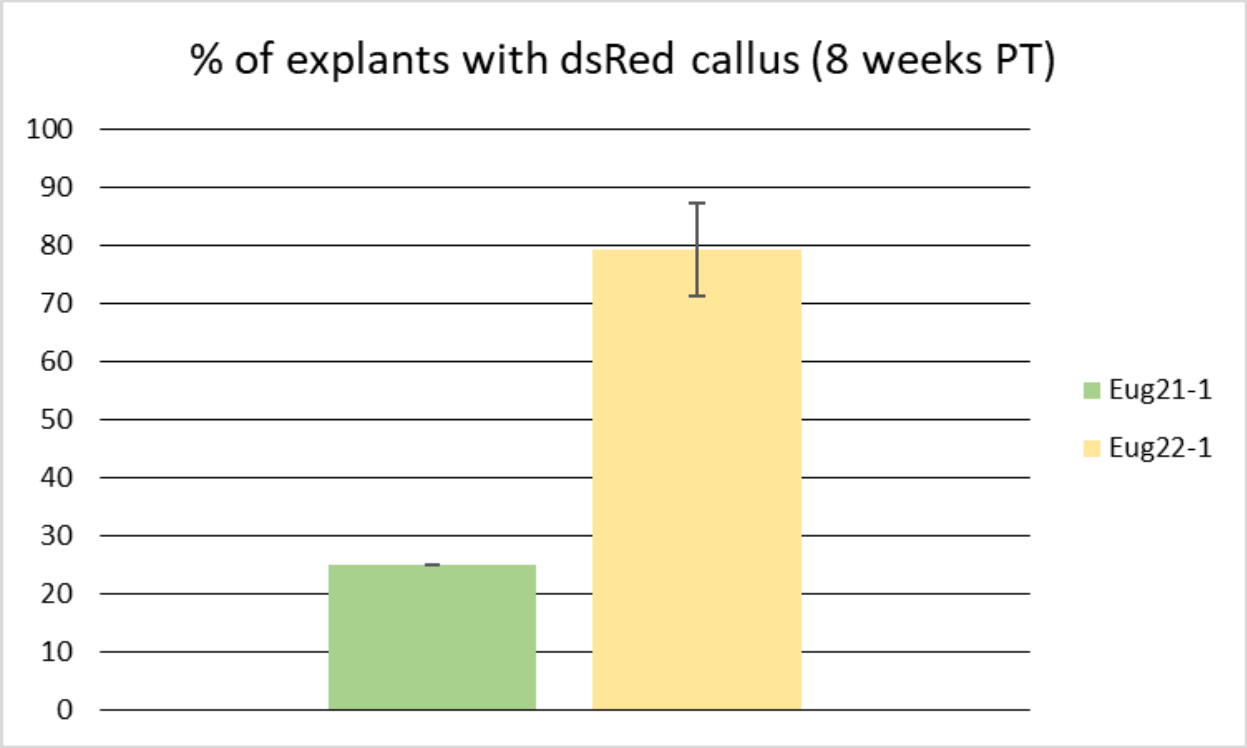


Figure A.3: : Comparison of dsRed callus frequencies in *E. urophylla x grandis* genotypes 'Eug21-1' and 'Eug22-1' transformed with 1x35S:*Eg*GRF5-GIF1. Data is from 8-weeks post-transformation (PT). Empty vector not shown due to failed transformation. Error bars = standard error.

>P. trichocarpa|GRF4-GIF1

atgaatagtggtggtgcaggaggggttggggttgaaggaggaggtggtggagcagcggggatggcagctgggggaatgggg
acagcagcgatgacaatgaggtcaccatttacagtgtcacagtggcaagaactggaacatcaagctttgatctataagtacatggt
ggcaggtctgcctgttccacctgatcttgtgctccctattcagaggagctttgaatccatttctcatagattcttccaccatcccaccat
gagctattgcactttctatggcaagaaggtggatccggaaccaggtcgatgcaggaggaccgacggcaagaagtggaggtgctc
caaagatgcctacccagactccaagtactgtgagcgccacatgcaccgtggccgcaaccgttcaagaaagcctgtggaatcacaa
accatgacacagtcatcgtccaccgtgacatcactgactgttacaggaagcagcagtggaactggaagcttccagaaccttccatt
gcacacatatagcaatccccagggcactgcttctggaactaaccaatcatattatcatatgaactccattccctacggaatcccaacc
aaagattacaggtatcttcaagaacttacgcctgaaggtggggagcatagcttcttgtctgaagcctcaggaagcaacaaggggct
tcagatagactcacagctggacaatgcatggtctttgatgcaatccagagtctcatcattccccacagagaaatcaactgaaaactc
gatgttgcaaagtaatcatccccagcattcatttttcagtagtgatttcaccaccagggaatctgtgaaacaggacgggcagtctctt
cgacccttctttgatgagtggcctaaaaaccgagatgcctggtctggcctcgagaatgatagttccaaccagacctcattctctacaa
cgcagctgtcgatatccattccaatggcctcatctgacttctccacaagttgtcgttctccacgagataacgcggcggccgccatgca
acagcacctgatgcagatgcagcccatgatggcagcctattaccccagcaacgtcactactgatcatattcaacagtatctggacga
aaacaagtcattgattttgaagattgttgagagccagaattcagggaaactcagtgagtgtgcagagaaccaagcaagactgcaa
caaaatctcatgtacttggctgcaattgctgattgtcagccccaaccacctaccatgcatgcccagttcccttccagcggcattatgca
gccaggagcacattacatgcagcatcaacaagctcaacagatgacaccacaagcccttatggctgcacgctcttctatgctgcagt
atgctcaacagccattctcagcgcttcaacaacagcaagccttacacagccagctcggcatgagctctggtggaagcgcaggactt
catatgatgcaaagcgaggctaacactgcaggaggcagtggagctcttggtgctggacgatttcctgattttggcatggatgcctc
cagtagaggaatcgcaagtgggagcaagcaagatattcggagtgcagggtctagtgaagggcgaggaggaagctctggaggc
cagggtggtgatggaggtgaaacccttacttgaaatctgctga

Figure A.4: **Sequence of *P. trichocarpa* GRF4-GIF1 chimera produced in this study.** Gray = *P. trichocarpa* GRF4, green = alanine bridge linker, blue = *P. trichocarpa* GIF1.

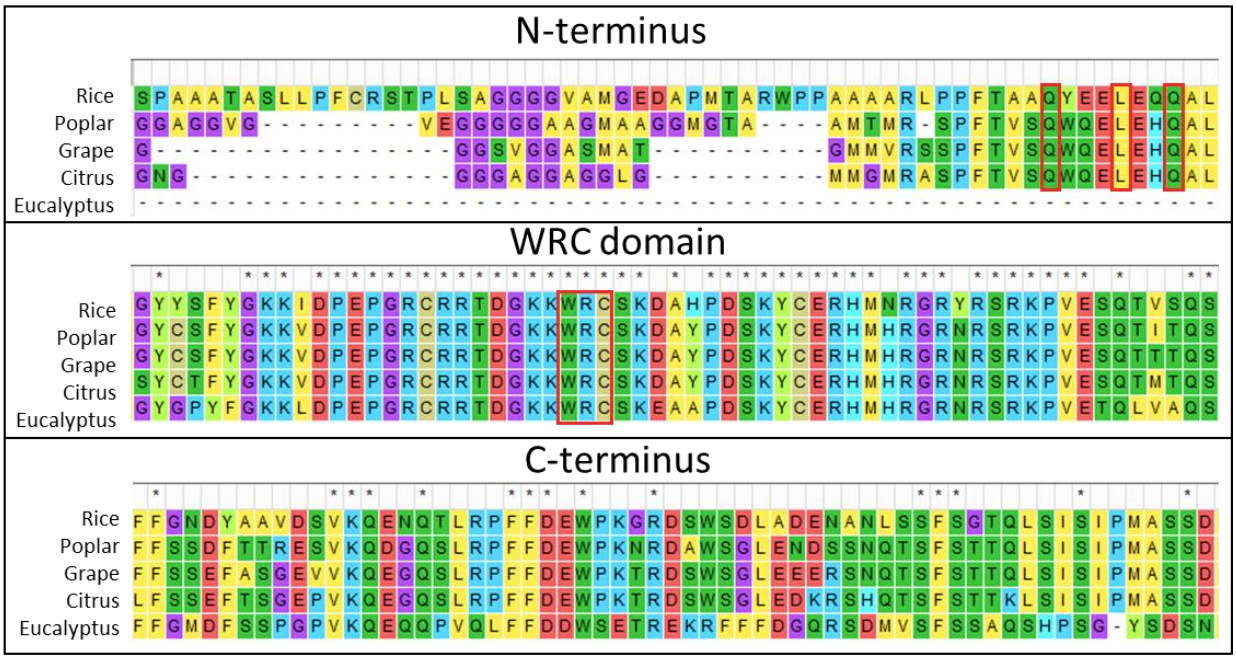


Figure A.5: Protein alignment of GRFs used in this this study (plus outgroup – rice). Amino acid sequences of *Oryza sativa* GRF4 (Os02g47280), *Populus trichocarpa* GRF4 (Potri.013G077500), *Vitis vinifera* GRF4 (VIT_216s0039g01450), *Citrus clementina* GRF4 (Ciclev10032065m.g), and *Eucalyptus grandis* GRF5 (Eucgr.F04420). Top: N-terminus with QLQ domains in all but *E. grandis* GRF5 (in red boxes), Middle: highly conserved section of gene with conserved WRC domain shown (in red boxes), Bottom: C-terminus. Stars above sequence indicate 100% agreement. Alignment was done using the ClustalW algorithm in MEGA7 software.

Gene	Forward primer	Reverse primer
*Pt*GRF4-like	5’ cctgcaggaacactaaaagtagaagaaaaatgaatagtggtggtg	5’ aggtgctgttgcatggcggccgccgcgttatctcgtggaga
*Pt*GIF1-like	5’ gcggccgccatgcaacagcacct	5’ actagttcagttcccatcatcagcag

Table A.2: Primers used to clone *Populus trichocarpa* GRF4-like and GIF1-like genes from synthetic gene fragments. GRF4-like forward primer included a 5’ UTR. All primers include flanking restriction enzyme cut sites.

Gene	Forward primer	Reverse primer
*Eg*GRF5-like	5’ cctgcaggaacactaaaagtagaagaaaaatgagtgggagtgga	5’ ggcggccgccgccctgccatctttatggttctgagaataa
*Eg*GIF1-like	5’ gcggccgccatgcagcagcacctgatgca	5’ actagttcaattaccatcgtctgctgatttc

Table A.3: Primers used to clone *Eucalyptus grandis* GRF5-like and GIF1-like genes from synthetic gene fragments. GRF5-like forward primer included a 5’ UTR. All primers include flanking restriction enzyme cut sites.

Expected frequencies

Genotype	Control	*Citrus* rGRF-GIF	Totals
717-1B4	28.57	11.61	40.18
353-53	25.64	10.43	36.08
Totals	54.22	22.04	76.26

Observed frequencies

Genotype	Control	*Citrus* rGRF-GIF	Totals	P-value
717-1B4	19.99	20.19	40.18	< 0.01
353-53	34.23	1.85	36.08	
Totals	54.22	22.04	76.26	

Table A.4: Chi-squared contingency tables showing expected versus observed regeneration frequencies of *Citrus* rGRF-GIF and the empty vector control for the poplar genotypes tested. Numbers represent % explants with at least one shoot.

717-1B4

Construct	Dead	Living	Totals	p-value
Control	1	4	5	0.047
***Citrus* rGRF-GIF**	5	0	5	
Totals	6	4	10	

353-53

Construct	Dead	Living	Totals	p-value
Control	0	5	5	0.007
***Citrus* rGRF-GIF**	5	0	5	
Totals	5	5	10	

Table A.5: Fisher's exact test contingency tables showing mortality frequencies of *Citrus* rGRF-GIF and the empty vector control for the poplar genotypes tested. Numbers represent individual explants with at least one living shoot placed on EM.

Construct	717-1B4	353-53	Totals	p-value
Control	8	8	16	0.02
***Pt*GRF-GIF**	8	0	8	
Totals	16	8	24	

Table A.6: Rates of transgenic shoot mortality in two poplar genotypes transformed with *Pt*GRF-GIF. This contingency table was used in the Fisher's exact test comparing the mortality of genotypes 717-1B4 and 353-53 stably transformed with 1x35S:*Pt*GRF-GIF and the empty vector control.

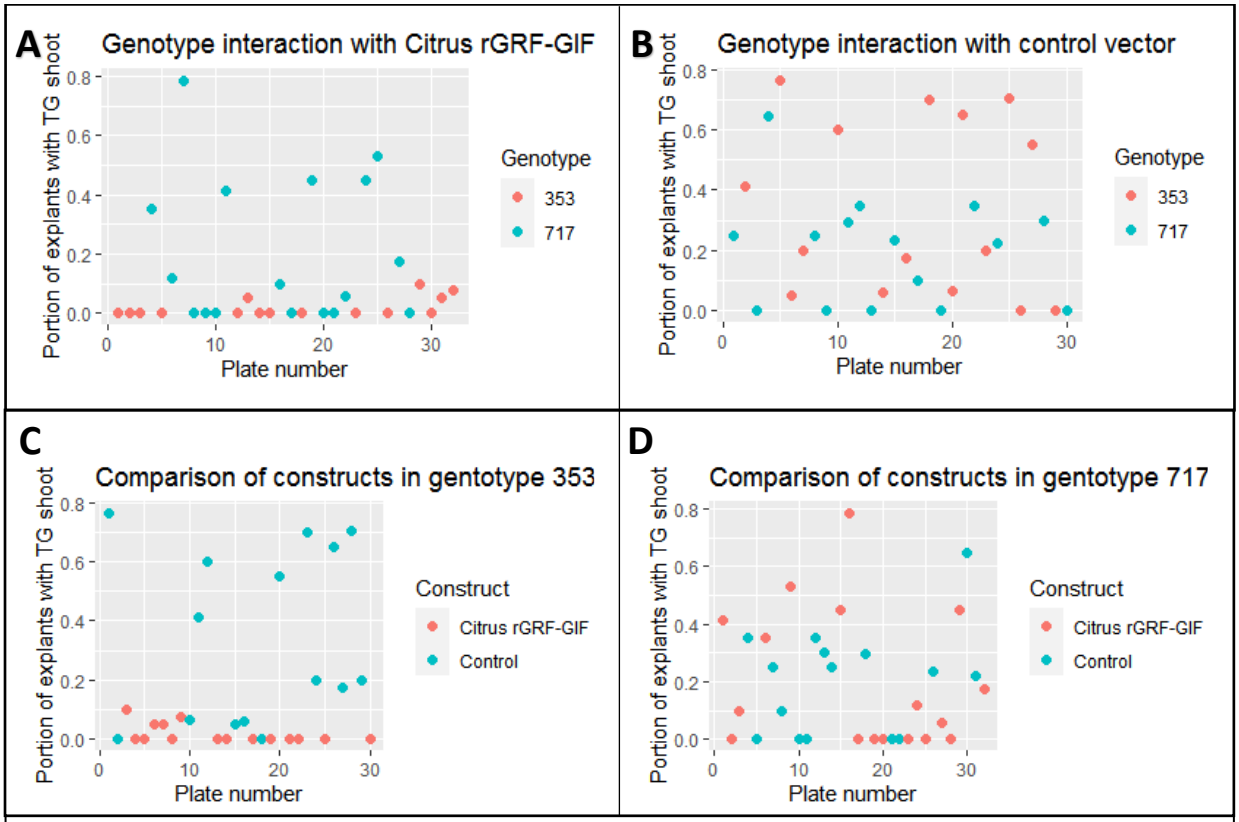


Figure A.6: Genotype interactions with the control and *Citrus* rGRF-GIF constructs. **(A)** Regeneration rates of hybrid poplar genotypes 353-53 and 717-1B4 when overexpressing *Citrus r*GRF-GIF. **(B)** Regeneration rates of the same genotypes when expressing the control vector (*hpt* + dsRed2). **(C)** Comparison of *Citrus r*GRF-GIF and the control vector in 353-53. **(D)** Comparison of *Citrus r*GRF-GIF and the control vector in 717-1B4. Data points represent the portion of explants with ≥1 transgenic shoot ('TG shoot') per plate.

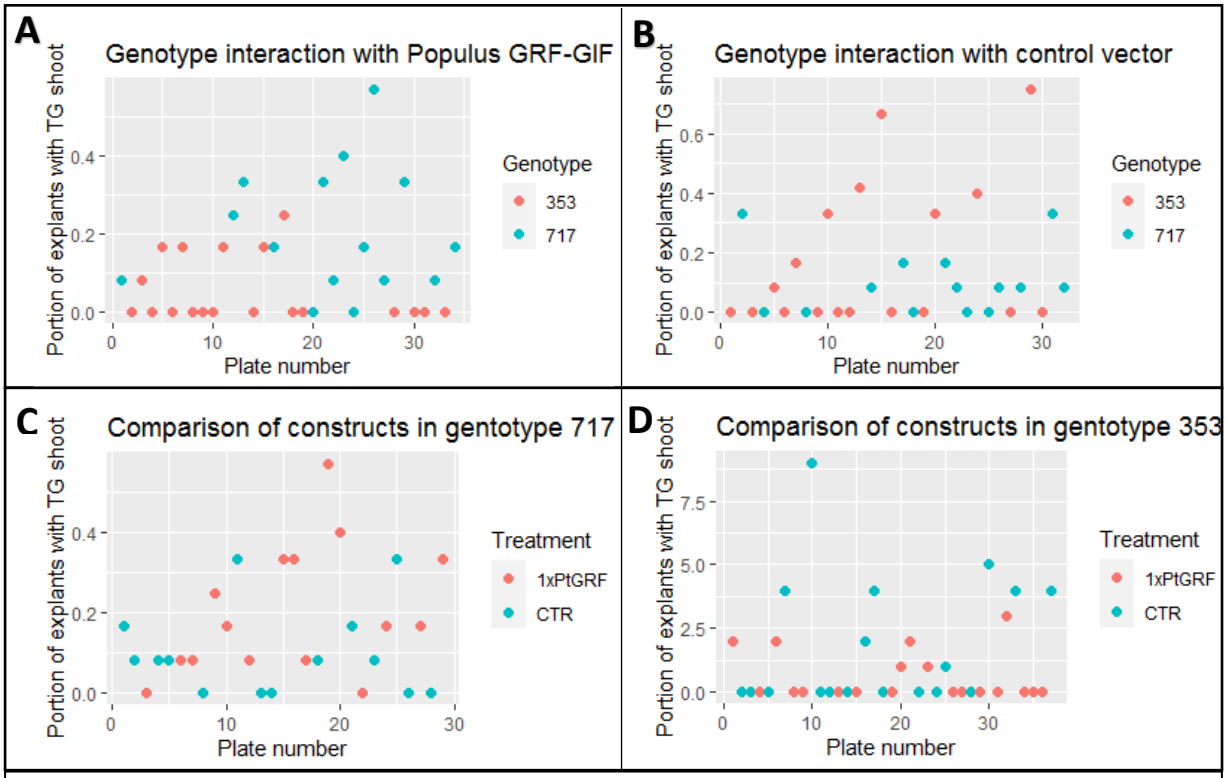


Figure A.7: Genotype interactions with the control and *Populus* GRF-GIF constructs **(A)** Regeneration rates of hybrid poplar genotypes 353-53 and 717-1B4 when overexpressing *Populus* GRF-GIF. **(B)** Regeneration rates of the same genotypes when expressing the control vector (*hpt* + dsRed2). **(C)** Comparison of *Populus* GRF-GIF and the control vector in 717-1B4. **(D)** Comparison of *Citrus r*GRF-GIF and the control vector in 353-53. Data points represent the portion of explants with ≥1 transgenic shoot ('TG shoot') per plate.

Figure A.8: Scatterplots showing Cook's distances for *Citrus* rGRF-GIF experiments as determined from a generalized linear model.

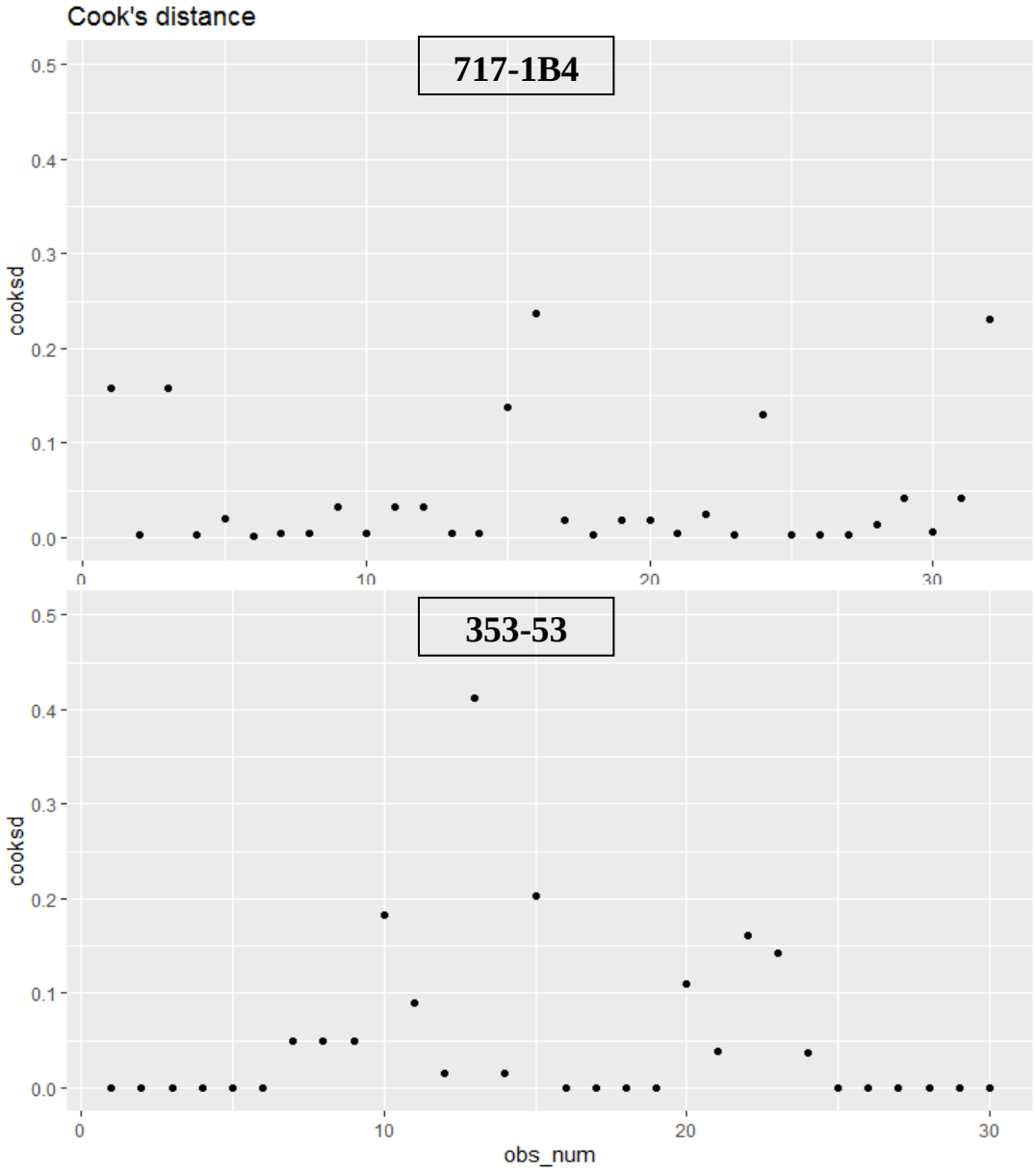


Figure A.9: Cook's distance scatterplots for 717-1B4 ectopic root frequency data before and after removal of the influential outlier (circled in magenta).

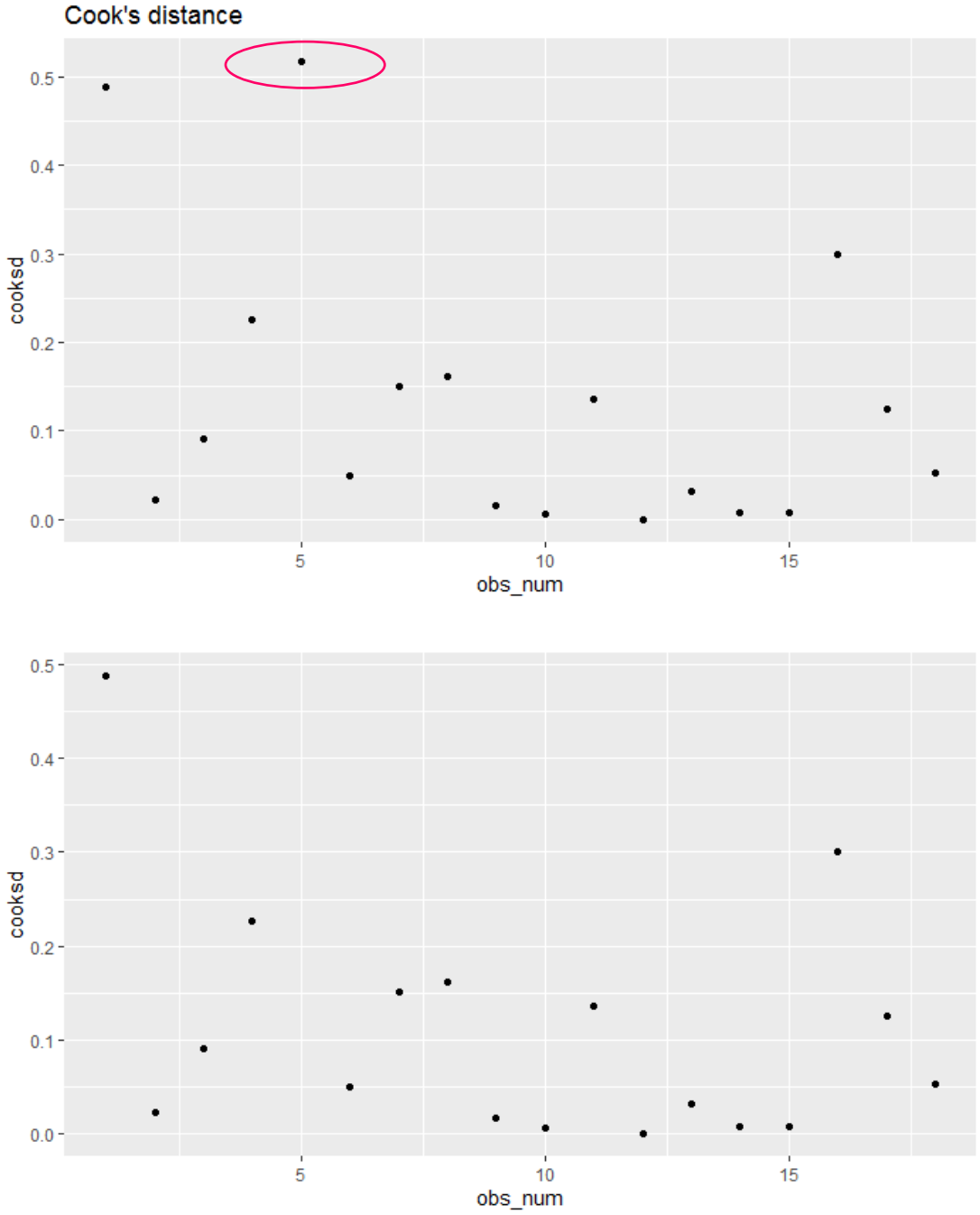


Figure A.10: Diagnostic plots from the 717-1B4 ectopic root data indicated the assumptions of normality (A-B) and equal variance (C) were supported.

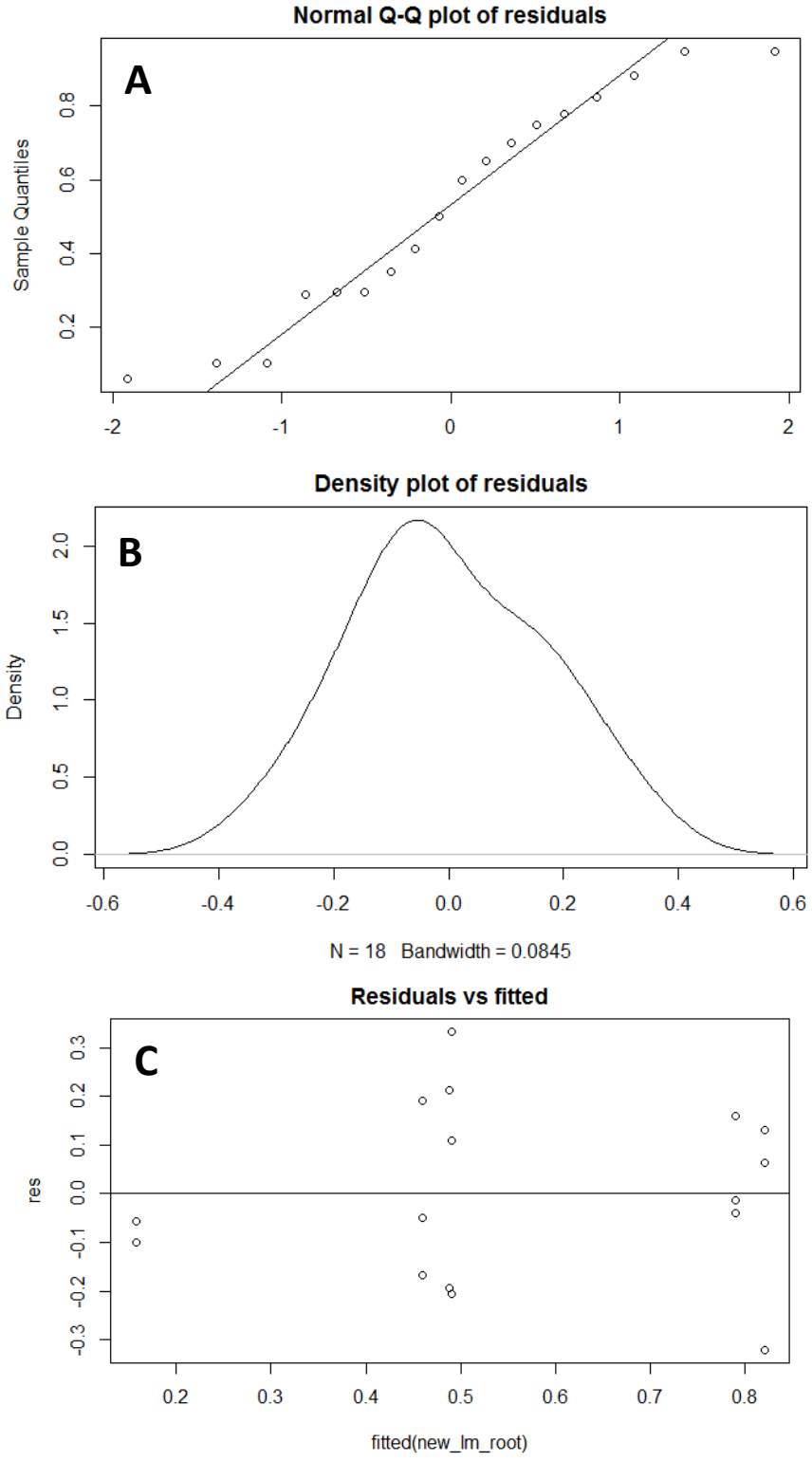


Figure A.11: Histograms showing the distributions of transgenic (TG) shoot prevalence data from the *Citrus* rGRF-GIF experiments.

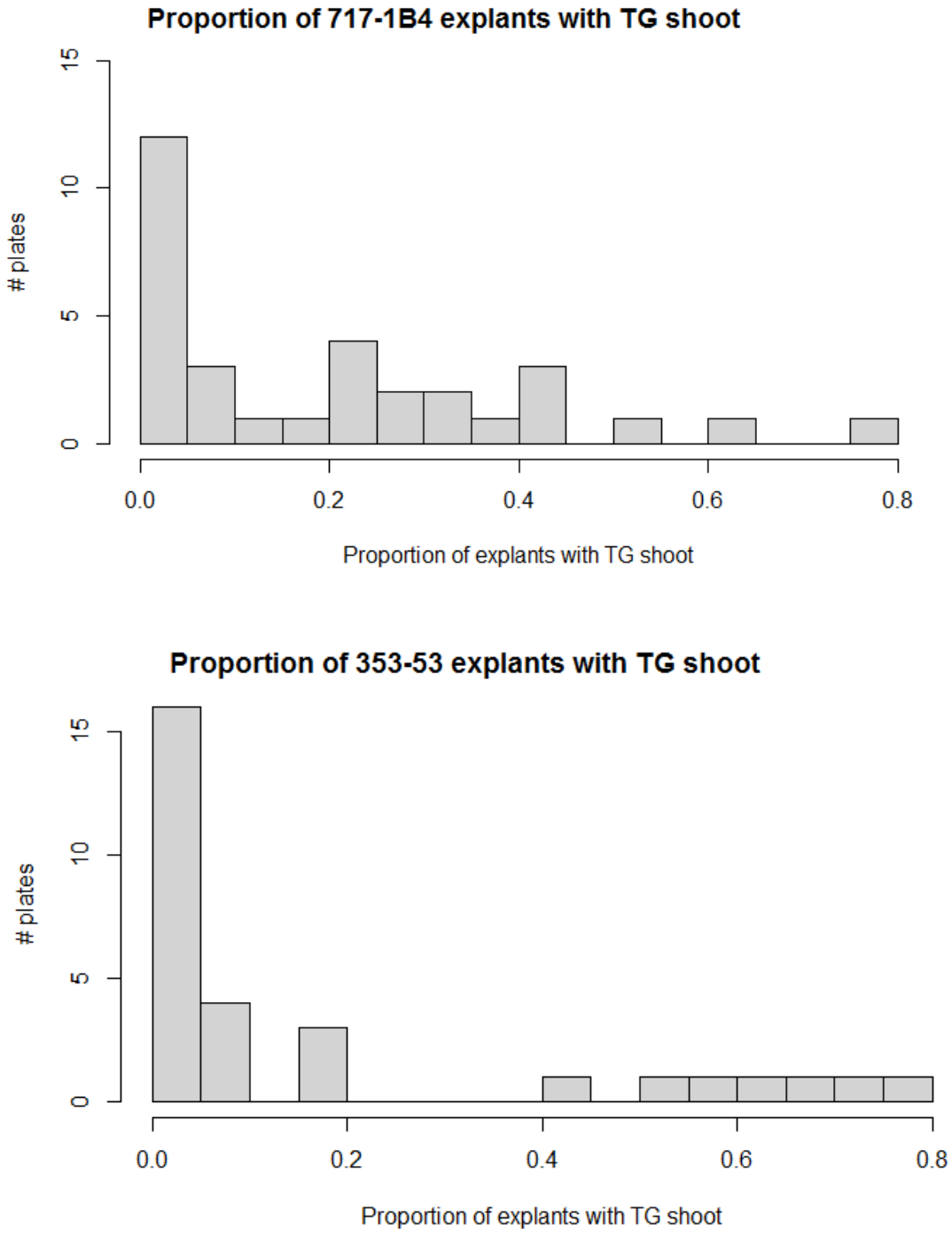


Figure A.12: Scatterplots of transgenic callus frequency data from *Pt*GRF-GIF experiments.

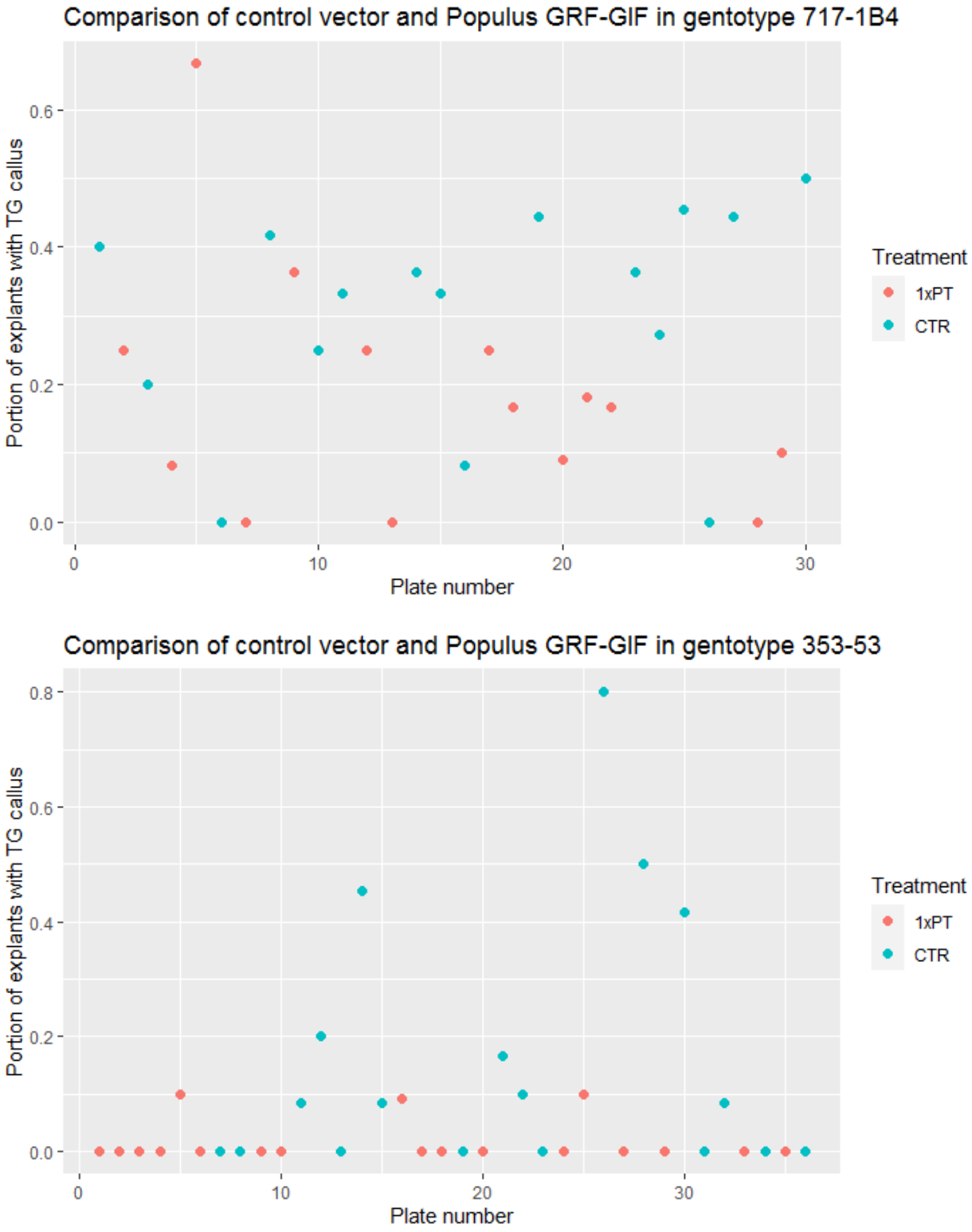


Figure A.13: Cook's distance scatterplots from the *Pt*GRF-GIF shoot regeneration data.

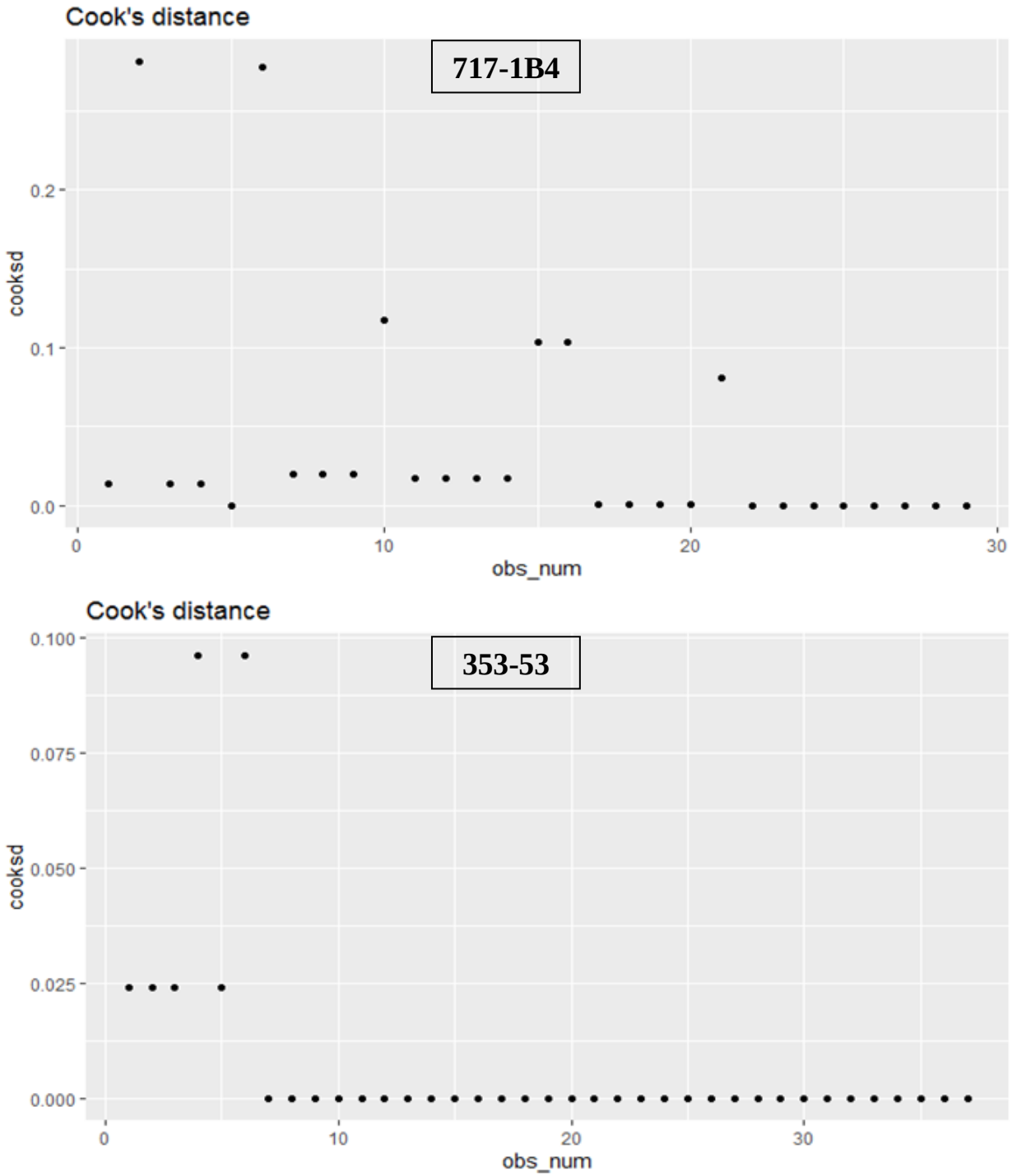
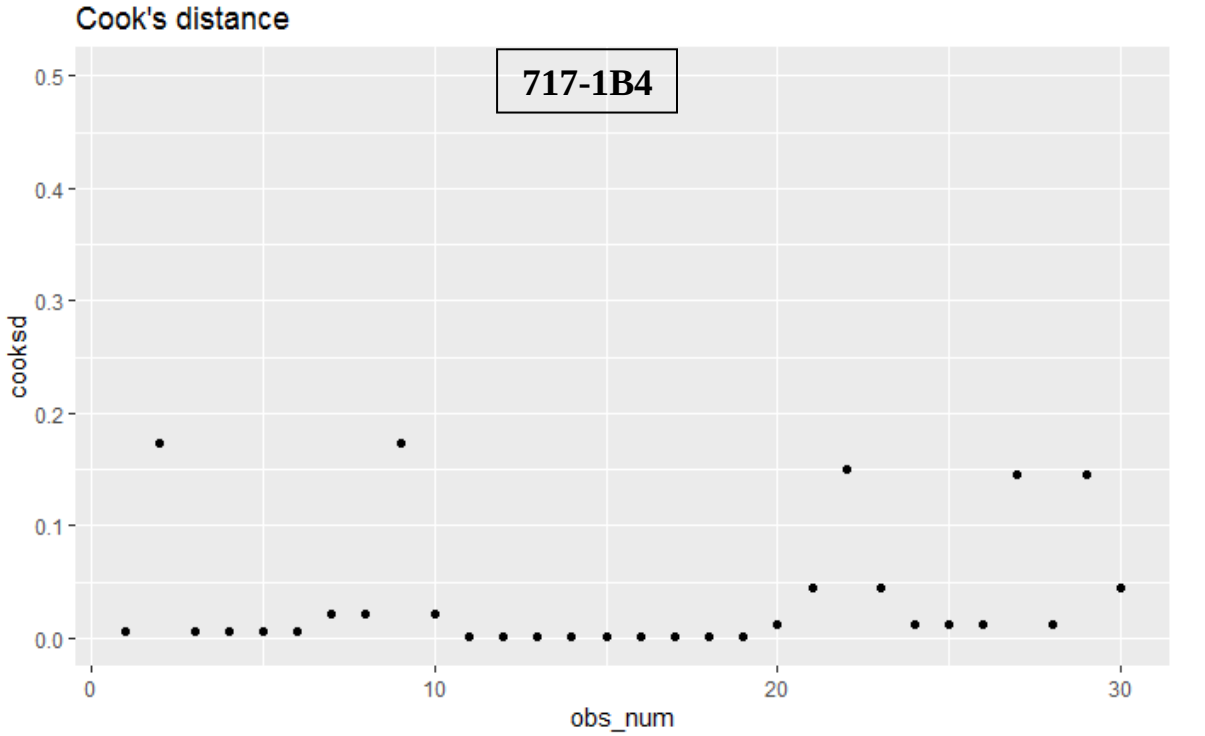


Figure A.14: Cook's distance scatterplots from the *Pt*GRF-GIF transgenic callus frequency data.



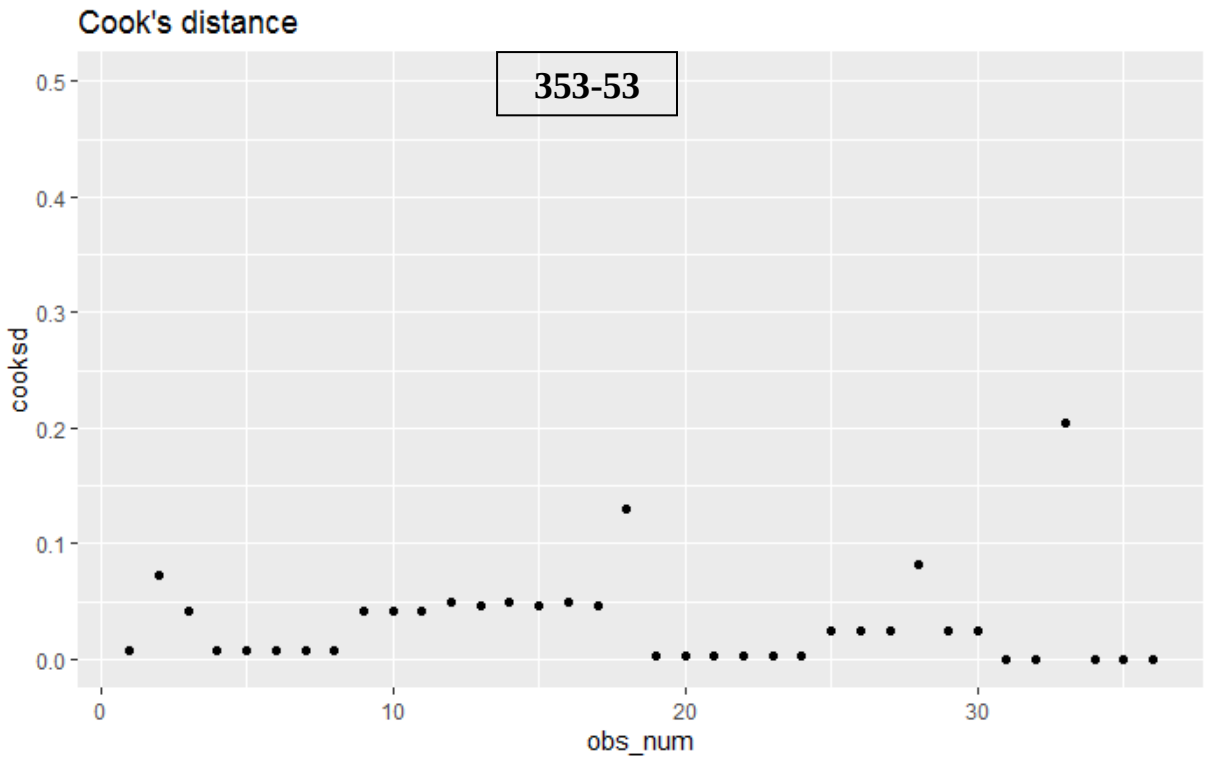
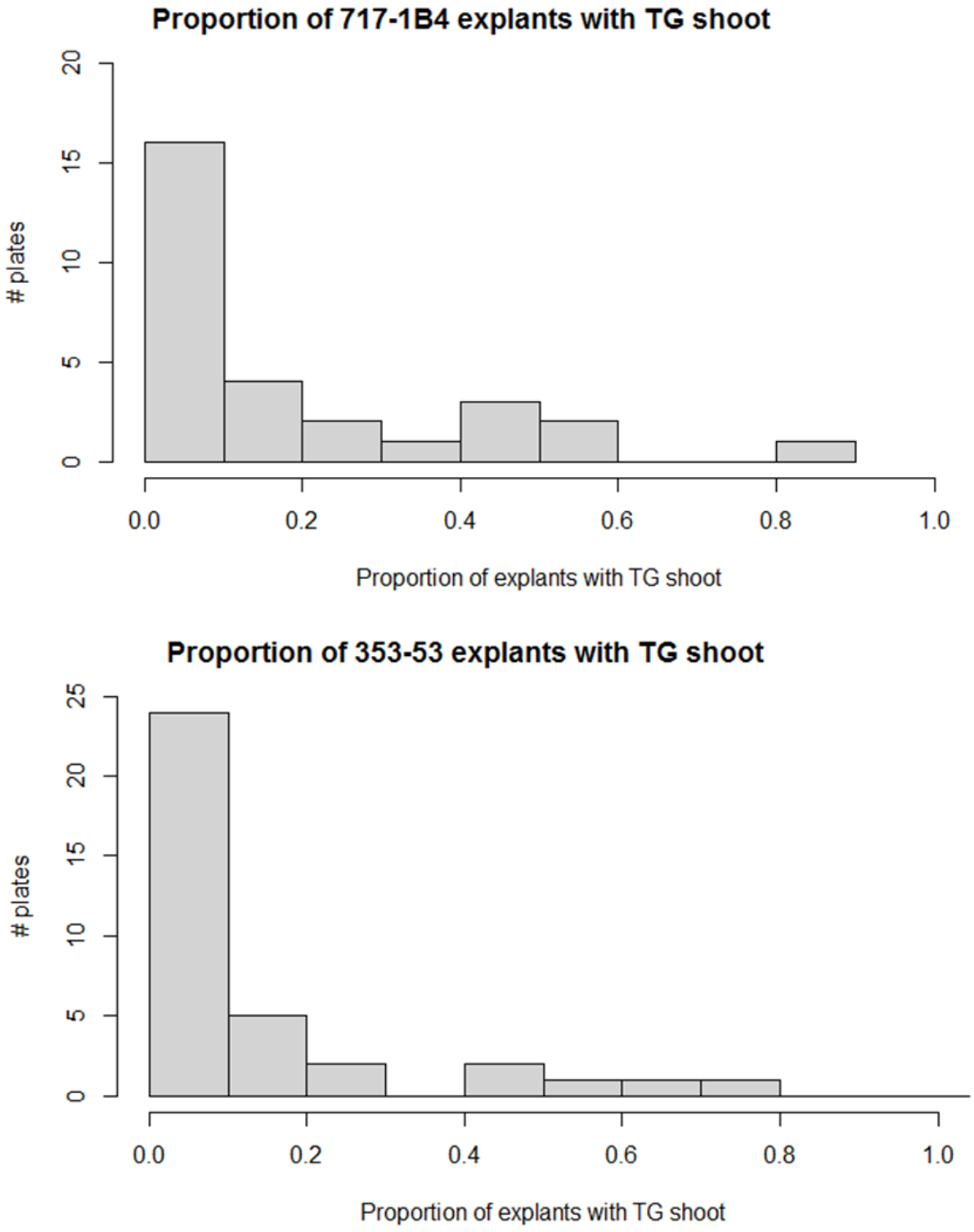


Figure A.15: Histograms showing distributions of transgenic (TG) shoot prevalence data for the *Pt*GRF-GIF experiments



Appendix B: Eucalypt transformation with *Citrus* r*GRF4-GIF1*

Eucalypt transformation method

Two eucalypt clones were used in this study: 'SP7' (*E. grandis x urophylla*) and 'Eug21-1' (*E. urophylla x grandis*). Leaves from *in-vitro* grown eucalypt plantlets (approx. 40-60 days old) with petioles are harvested and the leaf tips are removed. Leaves are washed in sterile ddH_2O for 1 hour to remove hydrophobic compounds from the leaf surface. Leaves are then placed on pre-culture callus-induction medium (PCIM) and left in the dark for one day. Following pre-culture, plants are inoculated with Agrobacterium containing a binary vector through wounding with a No. 10 scalpel blade dipped in spun-down and resuspended Agrobacterium cells (OD 2.0). After this, leaves are placed back onto PCIM for two days for co-cultivation in the dark. After this period, leaves are washed using the same protocol as described above and placed on eucalypt calli induction media (ECIM) containing appropriate antibiotics for one week and then once again placed in darkness. After one week, leaves will be subcultured onto eucalypt shoot induction medium (ESIM) containing appropriate antibiotics and placed in half-strength light until shoots form (2-3 months). A full protocol can be found in (Chauhan et al., 2014).

Eucalypt transformation with *Citrus* r*GRF4-GIF1*

In a smaller, replicated experiment, there was a moderate increase in transgenic callus formation in eucalypt clone SP7 when overexpressing *Citrus r*GRF-GIF (Table B.1). Of the explants transformed with the control vector, 28.8% produced transgenic callus compared with 32.9% of explants transformed with the *Citrus r*GRF-GIF vector. In a non-replicated experiment with clone Eug21-1, *Citrus r*GRF-GIF increased the prevalence of transgenic callus formation from 25% in the control vector group to 58.3%. Due to the small effect in SP7 and small sample size of Eug21-1, neither of these results is significant. However, the callus of the *Citrus r*GRF-GIF explants was visually observed to be much larger than that of the control explants (Figure B.2).

Table B.1: Details and data from the *Citrus* rGRF-GIF experiments in eucalypt. P-values calculated using Kruskal-Wallis non-parametric ANOVA.

Genotype	No. independent replicate experiments	Construct	Avg. No. explant/ replicate	Avg. plates/ replicate (20 grid)	Avg. No. explant with transgenic shoot(s)/ plate	Regen. Efficiency (total callus/ total explants)	% change vs control (P-value)
'SP7'	3	Control	23	2	3.5	28.8%	+12.5% (0.93)
		GRF-GIF	24	2	3.8	32.9%	
'Eug21-1'	1	Control	24	2	3.0	25.0%	+57.1% (0.12)
		GRF-GIF	24	2	7.0	58.3%	

Figure B.1: Bar charts comparing the percentage of explants produced transgenic calli 12 weeks PT in two eucalypt clones. Error bars = standard error.

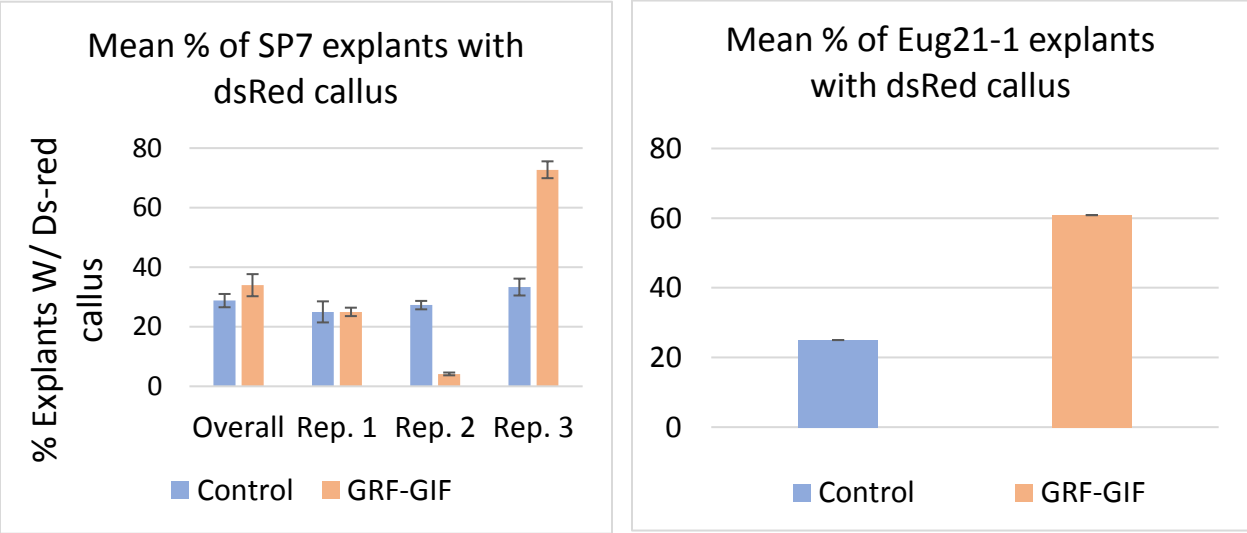
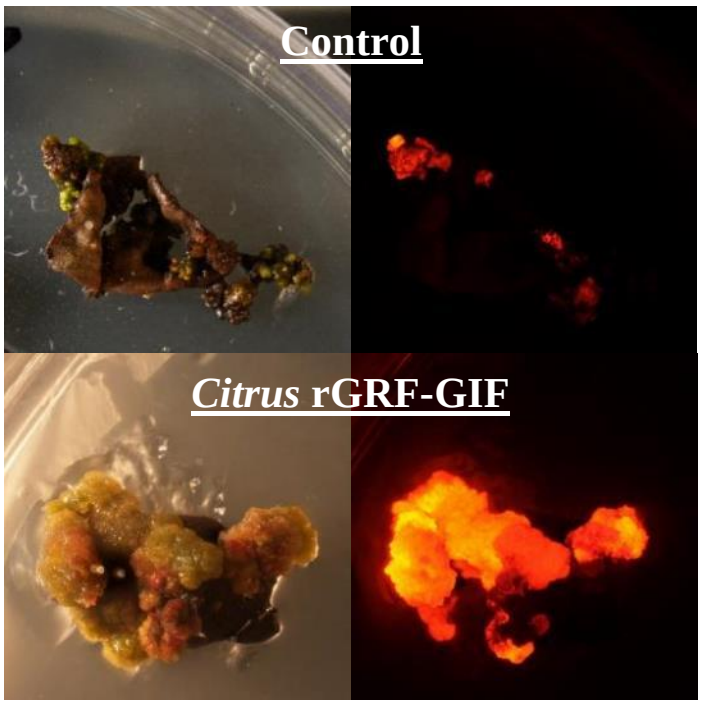
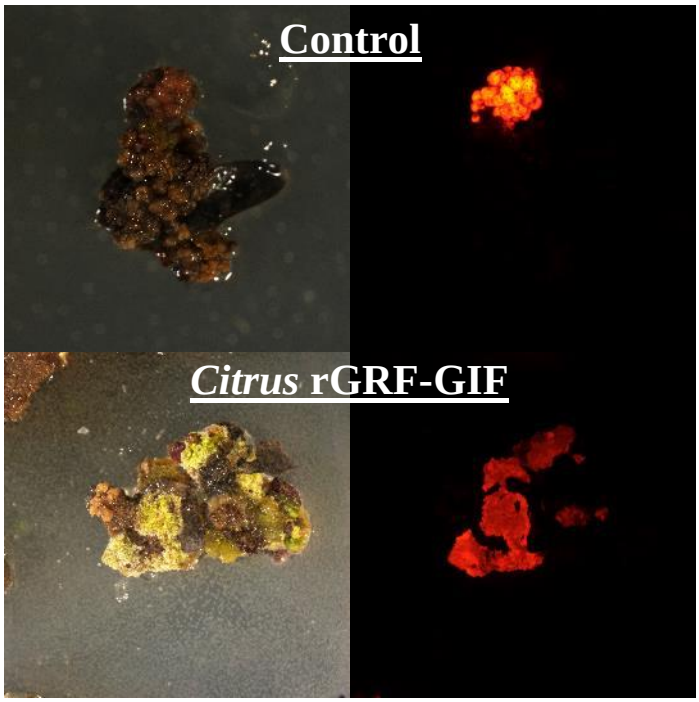


Figure B.2: Brightfield and fluorescent (dsRed) pictures taken 12 weeks PT of eucalypt clones ‘SP7’ and ‘Eug21-1’ show difference between rGRF-GIF and empty vector callus size and dsRed expression level.

‘SP7’



‘Eug21-1’



Appendix C: Testing additional GRF-GIF chimeras from in hybrid poplar

In non-replicated experiments, poplar clones 717-1B4 and 353-53 were transformed with additional 2x35S:GRF4-GIF1 constructs from *Citus x clementina* and *Vitis vinifera* (see below).

From *Citrus x clementina:*

1. **miR396 partially-resistant:** One miRNA-396 binding site mutation
2. **miR396 sensitive (wild-type):** No miRNA-396 binding site mutations.

From *Vitis vinifera:*

1. **miR396 resistant:** Four miRNA-396 binding site mutations

C.1 Methods

The transformation protocol remained the same as detailed in Chapter 2. However, the CIM period was reduced by 7 days, from 21 to 14, to shorten the experiment times. Additionally, explants were scored using a novel scoring system which aimed to quantify the area occupied by transgenic (dsRed positive) shoots 5 weeks PT (Figure C.1).

C.2 Results

All GRF-GIF constructs reduced transgenic (TG) shoot regeneration in both genotypes. See figures C.2 & C.3 and tables C.1-3 below.

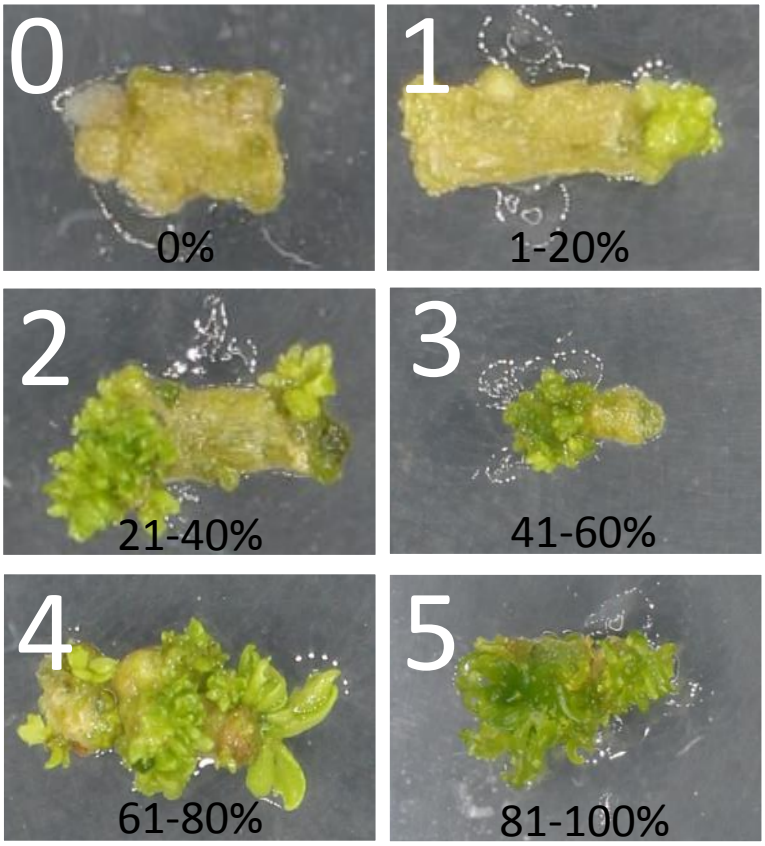


Figure C.1: Key used to score poplar explants in experiments detailed in Appendices C-D. Percentages represent explant area obscured by transgenic (dsRed positive) shoots. Numbers represent score (0-5).

Figure C.2: Whole-plate images of poplar stem explants showing differences in regeneration rates at 42 days post-transformation. Whole-plate images of poplar stem explants showing differences in regeneration rates between the empty vector control and various GRF-GIF chimeras at 5 weeks PT. Images were chosen to be representative of mean

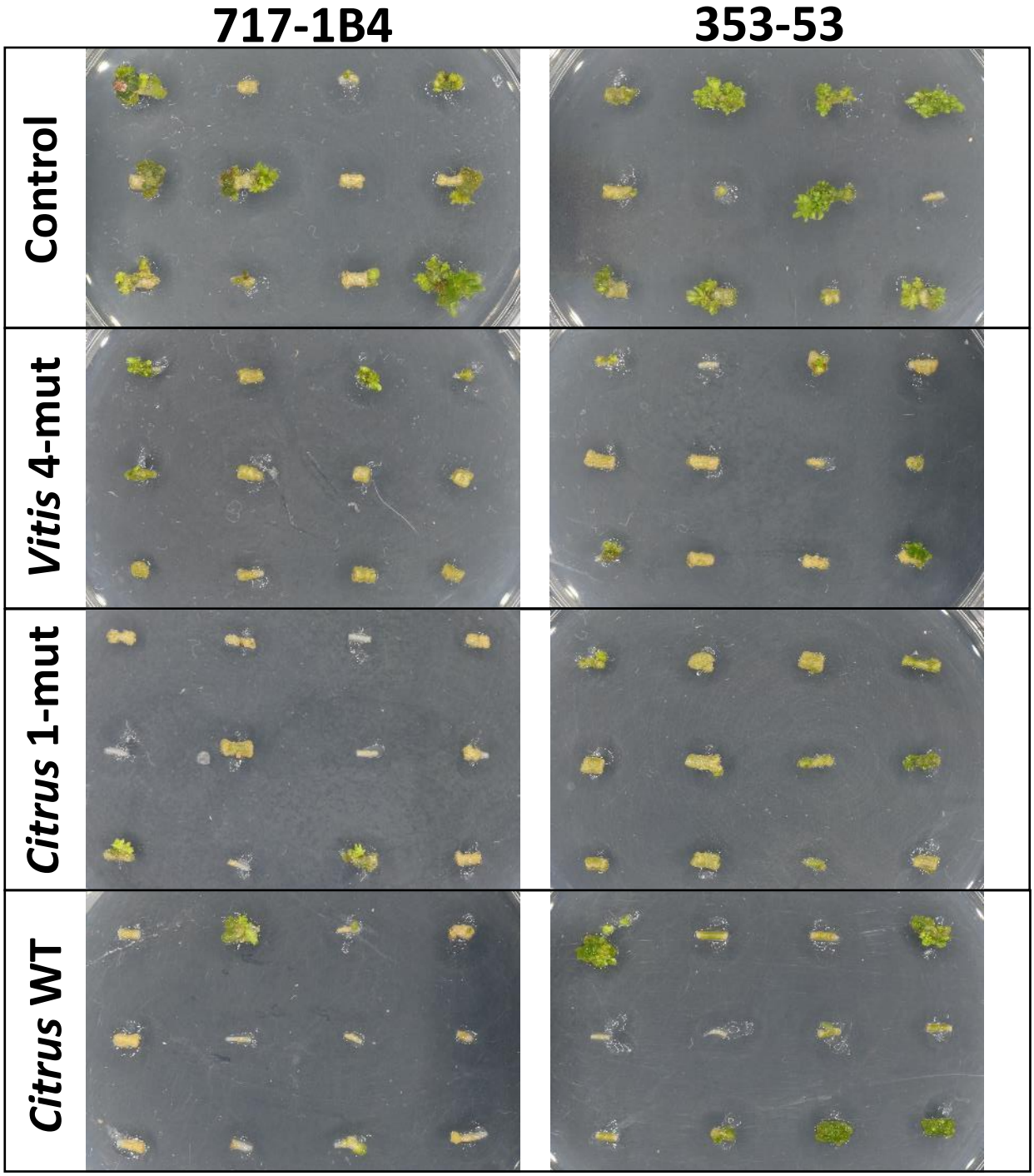


Figure C.3: Mean transgenic shoot scores of three distinct GRF-GIF constructs and an empty vector control construct. Data collected 5-weeks PT. Error bars = standard error.

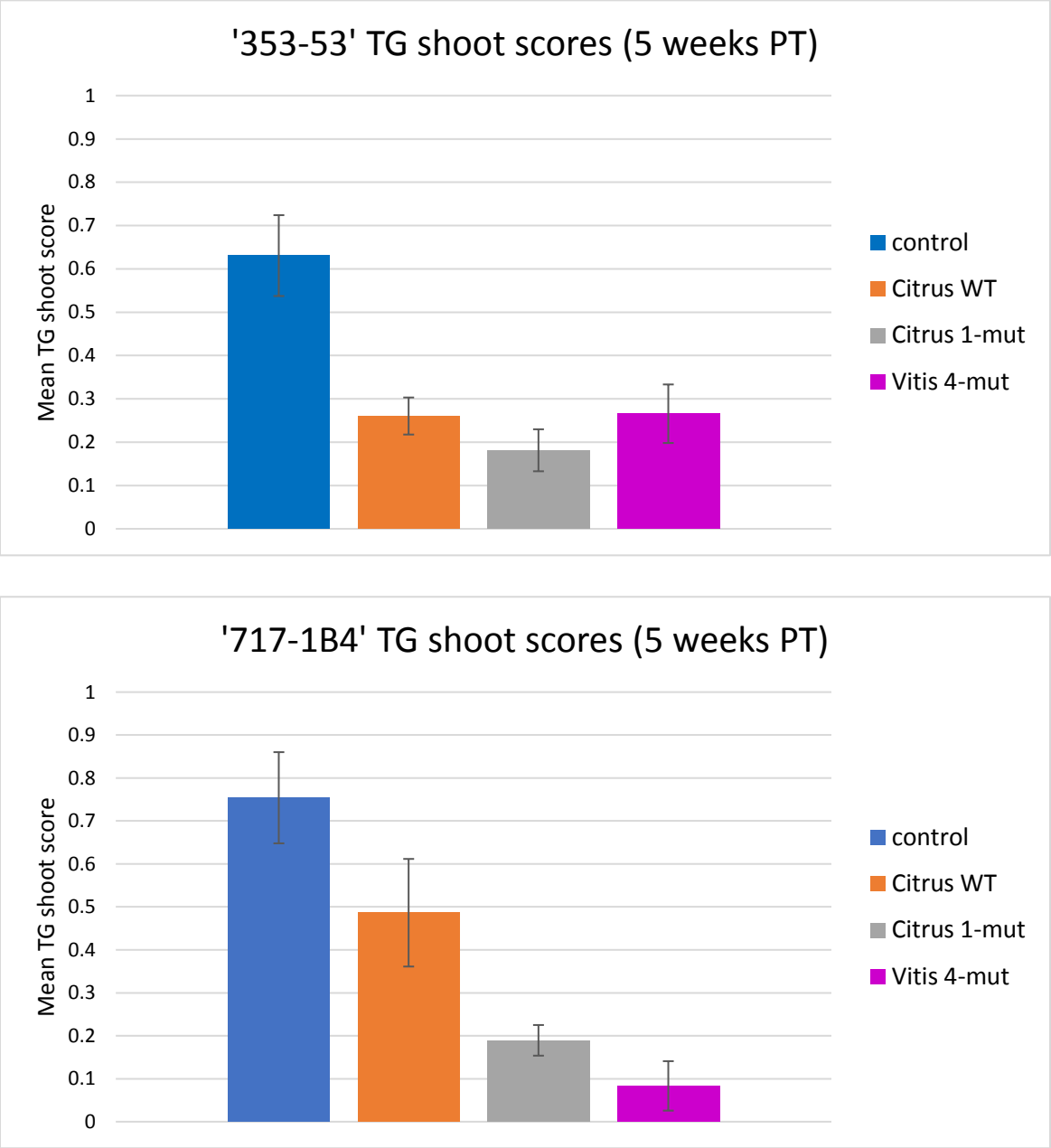


Table C.1: *Citrus* WT GRF-GIF overexpression experiment details and data.

Genotype	No. independent replicate experiments	Construct	Number of explant	Number of plates (12 grid)	Avg. No. explant with transgenic shoot(s)/ plate	Regen. Efficiency (total shoots/ total explants)	% change vs control (P-value)
'717-1B4'	1	Control	69	6	4.0	19.41%	-10.6% (0.17)
		GRF-GIF	74	7	3.3	18.58%	
'353-53'	1	Control	64	6	3.5	34.46%	-30.2% (0.03)
		GRF-GIF	83	7	2.7	1.75%	

Table C.2: *Citrus* 1-mutation GRF-GIF overexpression experiment details and data.

Genotype	No. independent replicate experiments	Construct	Number of explant	Number of plates (12 grid)	Avg. No. explant with transgenic shoot(s)/ plate	Regen. Efficiency (total shoots/ total explants)	% change vs control (P-value)
'717-1B4'	1	Control	80	7	5.4	47.5%	-76.0% (<0.01)
		GRF-GIF	123	11	1.3	11.4%	
'353-53'	1	Control	91	8	2.9	25.2%	-95.3% (0.01)
		GRF-GIF	81	7	0.1	1.2%	

Table C.3: *Vitis* 4-mutation GRF-GIF overexpression experiment details and data

Genotype	No. independent replicate experiments	Construct	Number of explant	Number of plates (12 grid)	Avg. No. explant with transgenic shoot(s)/ plate	Regen. Efficiency (total shoots/ total explants)	% change vs control (P-value)
'717-1B4'	1	Control	59	5	7.0	59.3%	-88.7% (<0.01)
		GRF-GIF	75	7	0.7	6.7%	
'353-53'	1	Control	73	7	3.4	32.9%	-51.4% (0.1)
		GRF-GIF	75	7	1.7	16.0%	

Table C.4: 2x35S:*Pt*GRF-GIF overexpression experiment details and data for *P. alba* clone '6K10.' P-value calculated using Fisher's exact test.

Genotype	No. independent replicate experiments	Construct	Number of explant	Number of plates (12 grid)	Avg. No. explant with transgenic shoot(s)/ plate	Regen. Efficiency (total shoots/ total explants)	% change vs control (P-value)
'6K10'	1	Control	39	4	0	0.0%	+100% (0.01)
		GRF-GIF	42	4	4.5	37.5%	

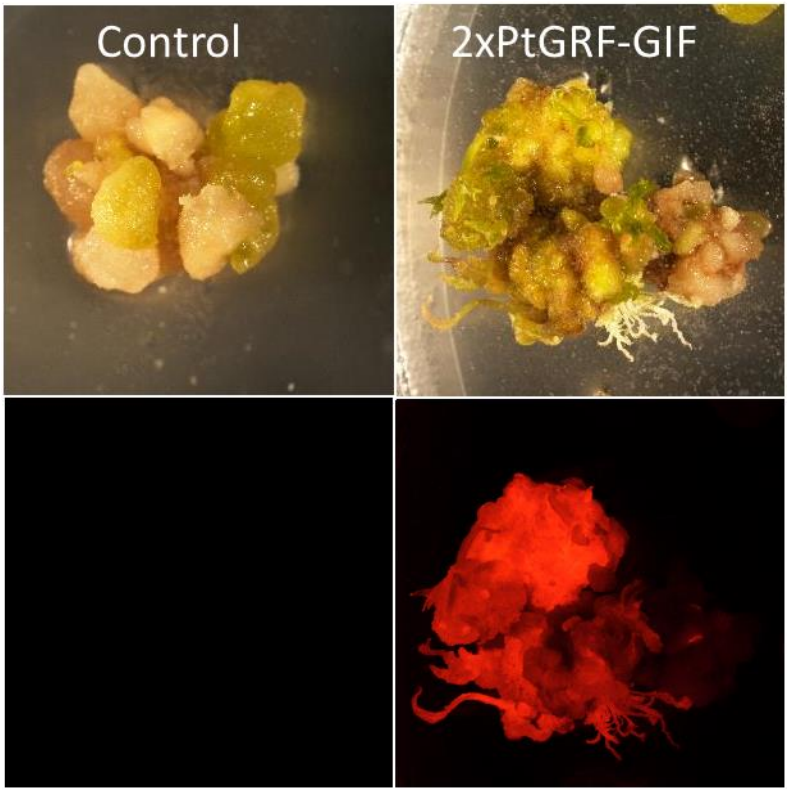


Figure C.4: Comparison of 6K10 explant morphology. Control = empty vector control. 2xPtGRF-GIF = 2x35S:*Pt*GRF-GIF. Images were taken 15 weeks PT. Images were chosen to be representative of mean morphology.

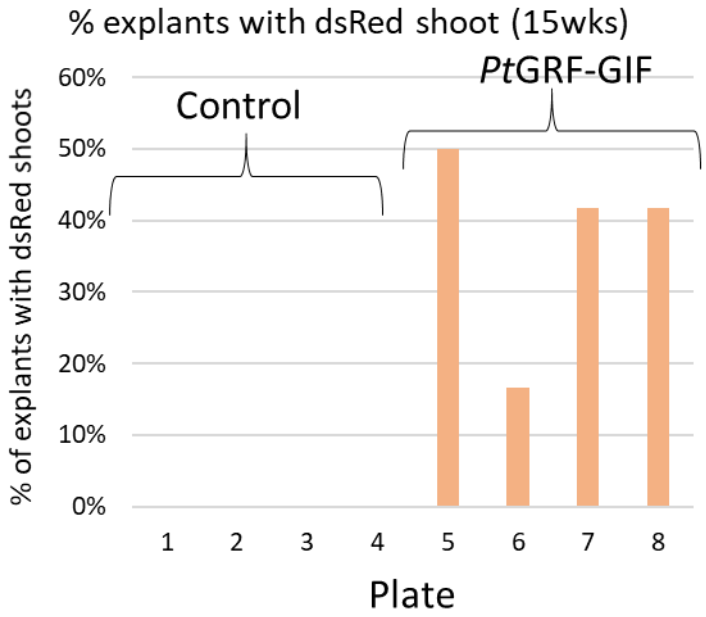


Figure C.5: 6K10 regeneration rates per plate (15-weeks PT). Control = empty vector control. 2xPtGRF-GIF = 2x35S:*Pt*GRF-GIF.

Construct	Plates with no TG shoot	Plates with ≥1 TG shoot	Totals	p-value
Control	4	0	4	0.01
2x*Pt*GRF-GIF	0	4	4	
Totals	4	4	8	

Table C.5: Fisher's exact test contingency tables showing transgenic (TG) shoot regeneration frequencies of *Pt*GRF-GIF and the empty vector control for the poplar genotype '6K10.' Numbers represent individual plates with at least one TG shoot.

Appendix D: Testing dexamethasone-activated GRF-GIF in poplar and eucalypt

D.2 Methods

A dexamethasone (DEX) activated miRNA-resistant GR (rat glucocorticoid domain)-GRF-GIF fusion (from *Vitis*) was tested in a non-replicated experiment with two poplar and two eucalypt clones.

Plant transformation and regeneration

Poplar

Poplar transformation followed the protocol outlined in Chapter 2. Three experimental groups were studied: an empty-vector control, GR-GRF-GIF without DEX (Mock treatment), and GR-GRF-GIF with DEX (treatment group). The mock treatment was included to study the leakiness of the control mechanism. DEX (dissolved in DMSO) was added to the callus-induction medium of the treatment group at a 5 µM final concentration. During this period, WPA (Lloyd & McCown, 1980) containing the same concentration of DEX was sprayed once weekly onto regenerating explants starting at day 7 and ending on day 21. The mock treatment group

was sprayed at the same intervals with WPA. Explants were subcultured onto DEX-free SIM on day 21. Transgenic (dsRed positive) shoots were scored according to the method detailed in Appendix C at 49 days post-transformation. This study was conducted in hybrid poplar genotypes 717-1B4 and 353-53.

Eucalypt

Eucalypt transformation followed the protocol outlined in Appendix B. The eucalypt experiments had the same three experimental groups as the poplar experiments. DEX (5 µM) was added to the CIM as well as to the SIM of the treatment group for a total of 56 days. The explants were also sprayed with DEX (same conc.) starting 7 days after the first subculture and continuing until 7 days before the subsequent subculture. The mock treatment group was sprayed at the same intervals with WPA. This process repeated after each subculture. Explants with transgenic callus and transgenic shoot primordia (Figure D.7) were counted at 56 days post-transformation. This study was conducted in hybrid eucalypt genotypes SP7 and Eug21-1 (detailed in Appendix B).

Statistical analysis

Data was analyzed using the Kruskal-Wallis rank sum test, which is a non-parametric alternative to one-way ANOVA (Kruskal & Wallis, 1952). Post-hoc analysis was conducted using a pairwise Wilcoxon rank sum test with corrections for multiple comparisons (Mann & Whitney, 1947).

D.2 Results

Poplar

The addition of DEX to the GR-GRF-GIF treatment group decreased transgenic shoot formation 49 days post-transformation in both poplar genotypes compared to the empty vector control and mock treatment. The difference between groups (control, mock, and Dex) was not statistically significant in 717-1B4 ($P = 0.21$) but was statistically significant in 353-53 ($P = 0.003$). The differences in regeneration scores in 353-53 between the empty vector control and the DEX treatment as well as the difference between the mock treatment and DEX treatment in

353-53 were both statistically significant (P = 0.002; P = 0.01). As expected, there was not a significant difference between the empty vector control and mock treatment groups (P = 0.61) (Figure D.3).

Eucalypt

There were no statistically-significant differences between groups for transgenic callus or primordia frequency (explants/plate) at day 56 post-transformation in either SP7 or Eug21-1 genotypes. However, there was an observable increase in both transgenic callus and primordia formation on Eug21-1 explants expressing the GR-GRF-GIF construct, regardless of dexamethasone addition. In fact, the mock treatment group produced transgenic callus and primordia at the highest frequency (Figure D.6). In SP7, only the dexamethasone-induced GRF-GIF explants had produced primordia at day 56 at a rate of 7.6% (see Table D.2). Explants were lost to contamination shortly after day 56.

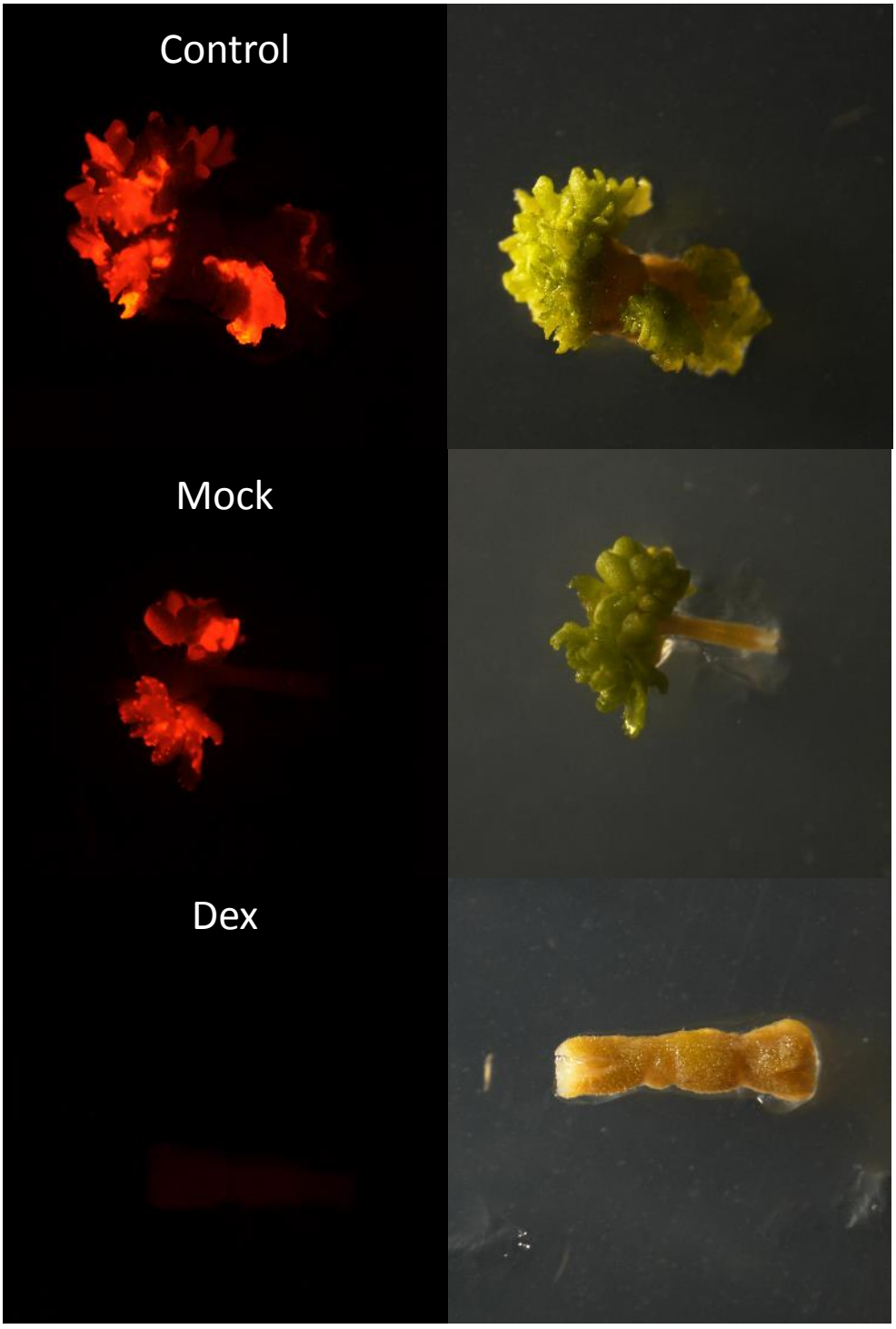


Figure D.1: Epifluorescent and bright field images of poplar '353-53' from the *Vitis* 4-mutation dexamethasone (Dex)-inducible GR-GRF-GIF experiment. Mock = 2x35S:GR-GRF-GIF without dexamethasone treatment. Images were chosen to be representative of phenotype.

Figure D.2: Epifluorescent and bright field images of poplar '717-1B4' from the *Vitis* 4-mutation dexamethasone (Dex)-inducible GR-GRF-GIF experiment. Mock = 2x35S:GR-GRF-GIF without dexamethasone treatment. Images were chosen to be representative of phenotype.

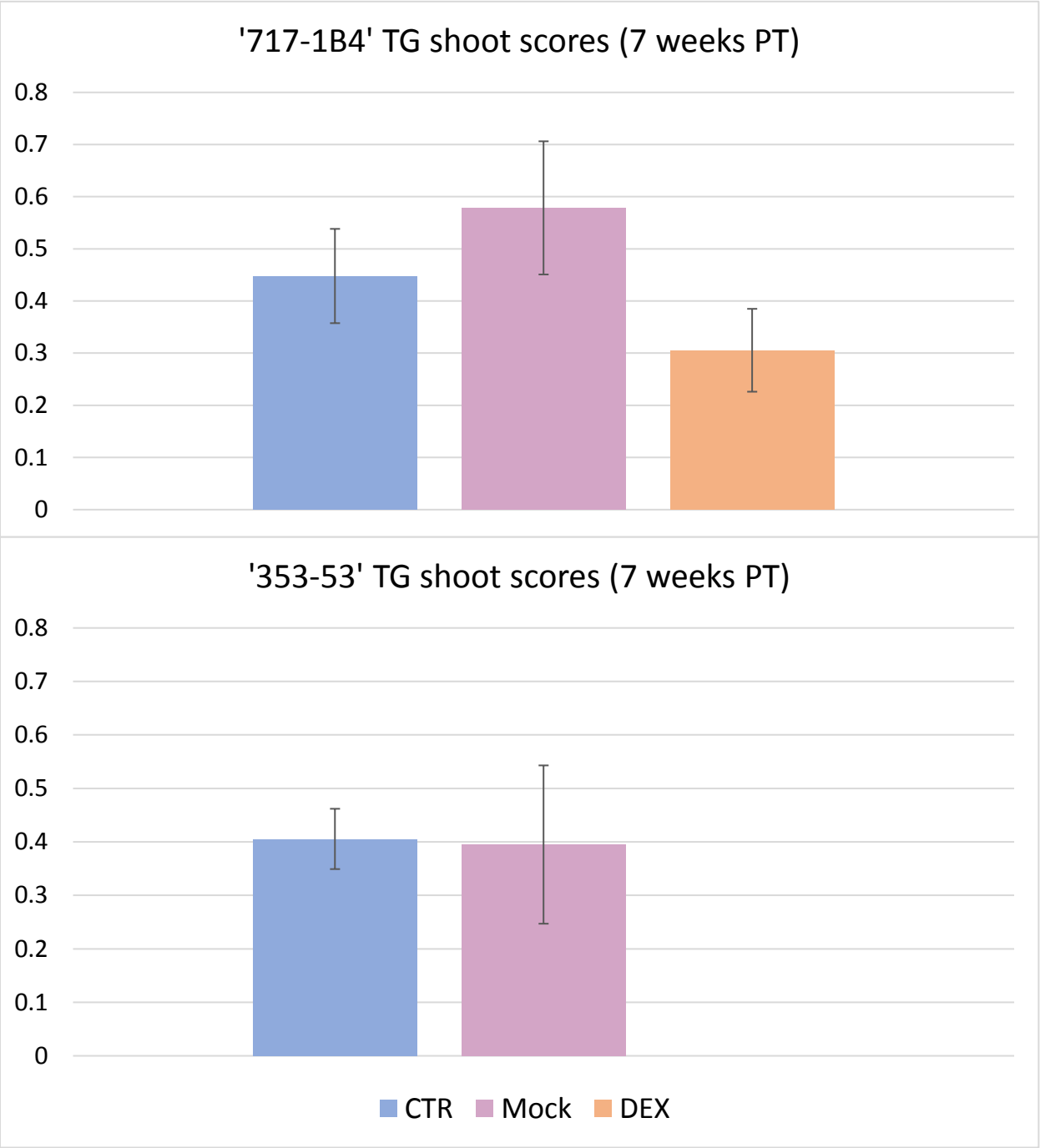


Figure D.3: Comparison of mean transgenic shoot scores of two poplar genotypes during dexamethasone-inducible *Vitis* 4-mutation GR-GRF-GIF experiments. CTR = empty vector control, MCK = mock treatment, DEX = dexamethasone treatment. TG = transgenic. 353-53 produced no shoots with DEX treatment.

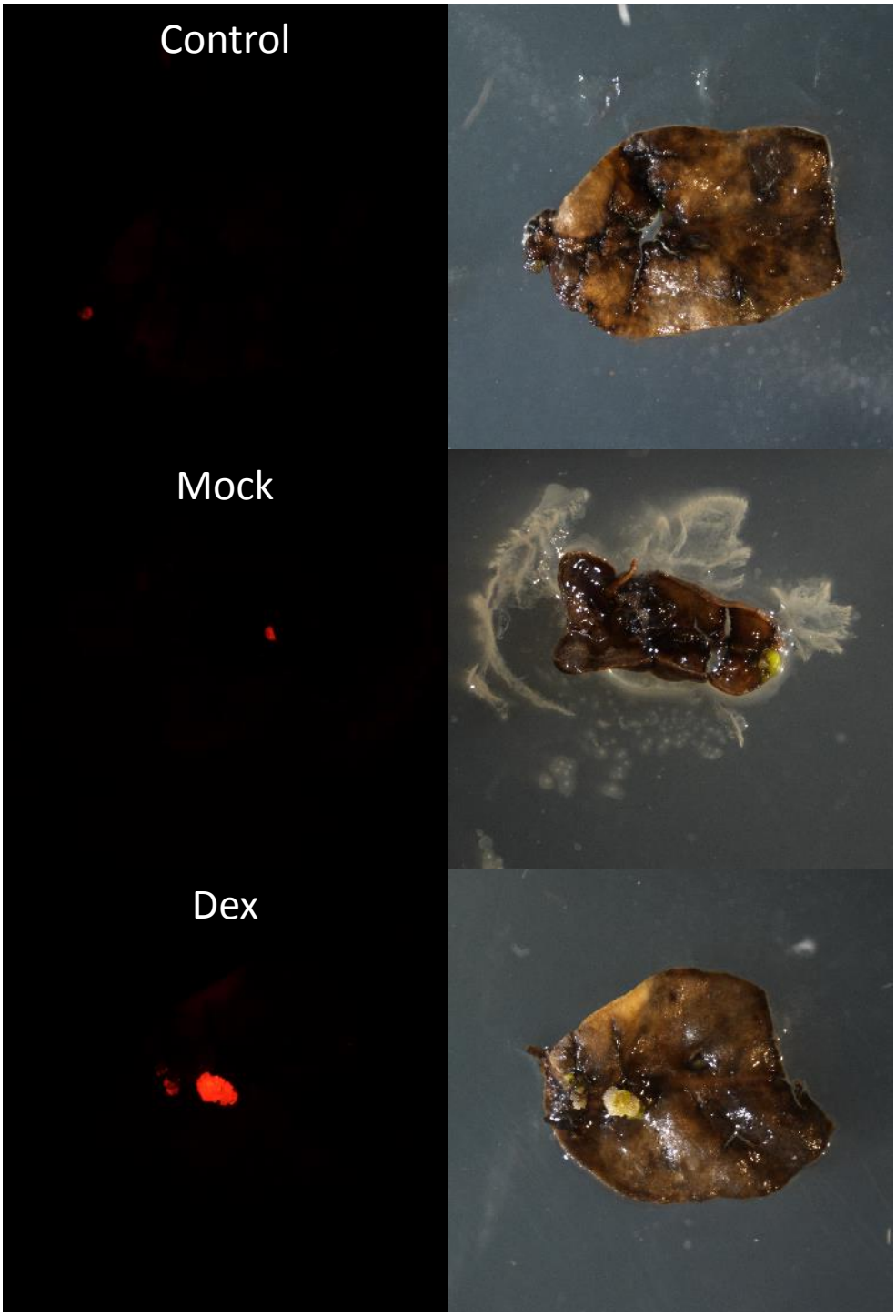


Figure D.4: Epifluorescent and bright field images of eucalypt 'SP7' from the *Vitis* 4-mutation dexamethasone (Dex)-inducible GR-GRF-GIF experiment. Images were chosen to be representative of phenotype.

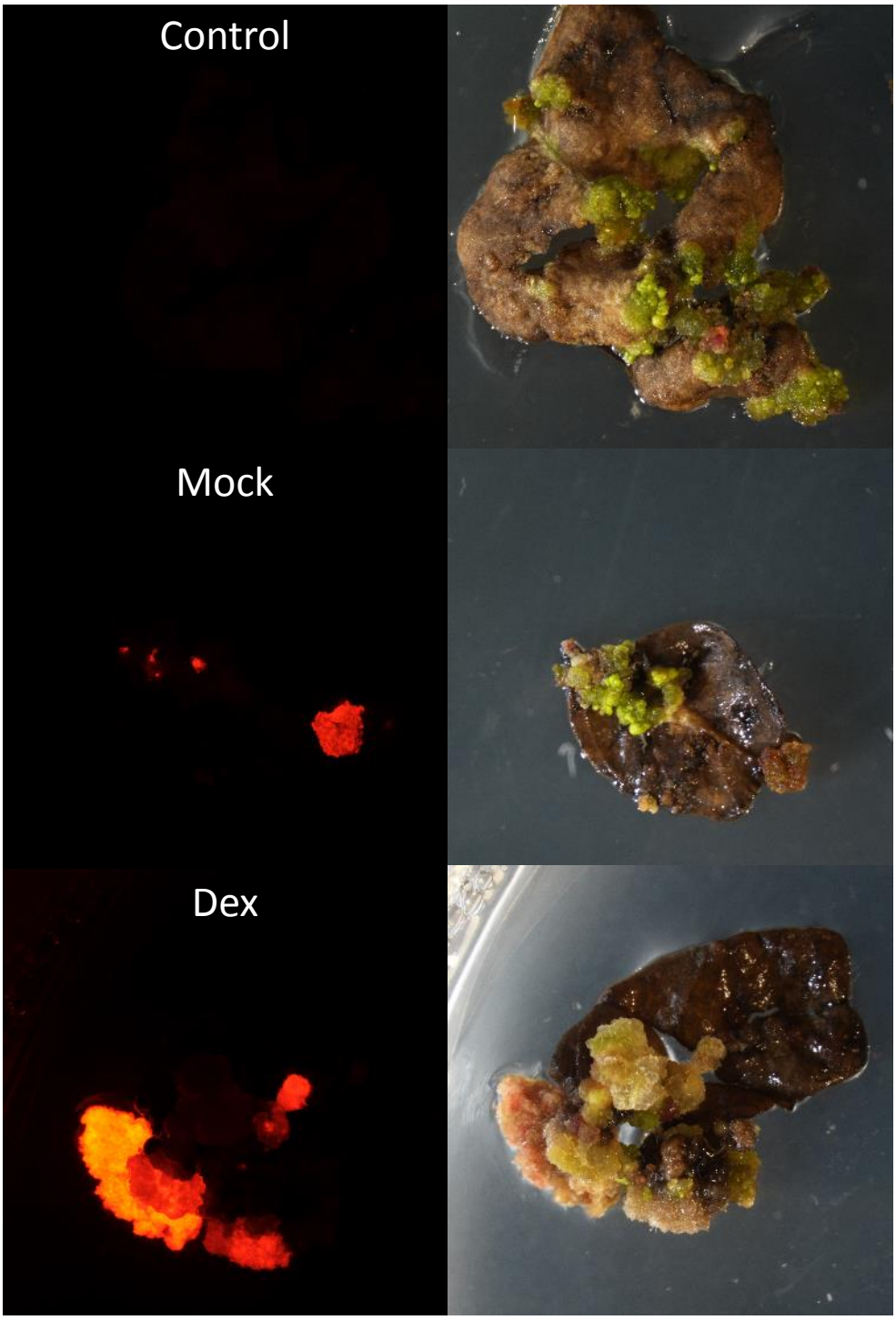


Figure D.5: Epifluorescent and bright field images of eucalypt 'Eug21-1' from the *Vitis* 4-mutation dexamethasone (Dex)-inducible GR-GRF-GIF experiment. Images were chosen to be representative of phenotype.

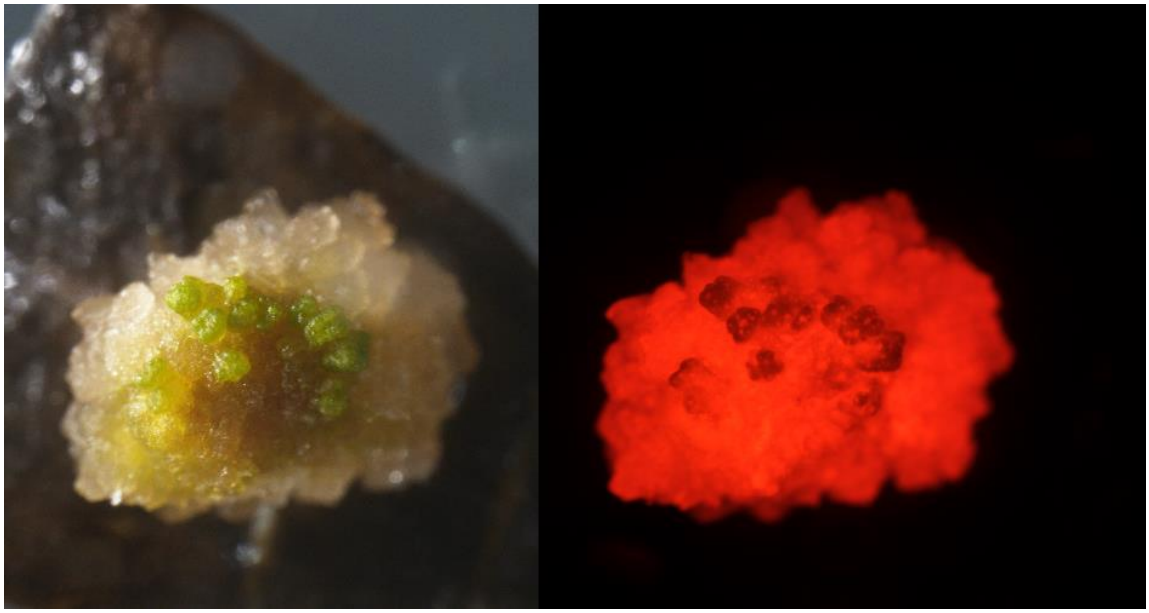


Figure D.6: Example of transgenic shoot primorida (in green) observed in eucalypt 'Eug21-1' 56 days post-transformation. These primordia came from a dex-treated

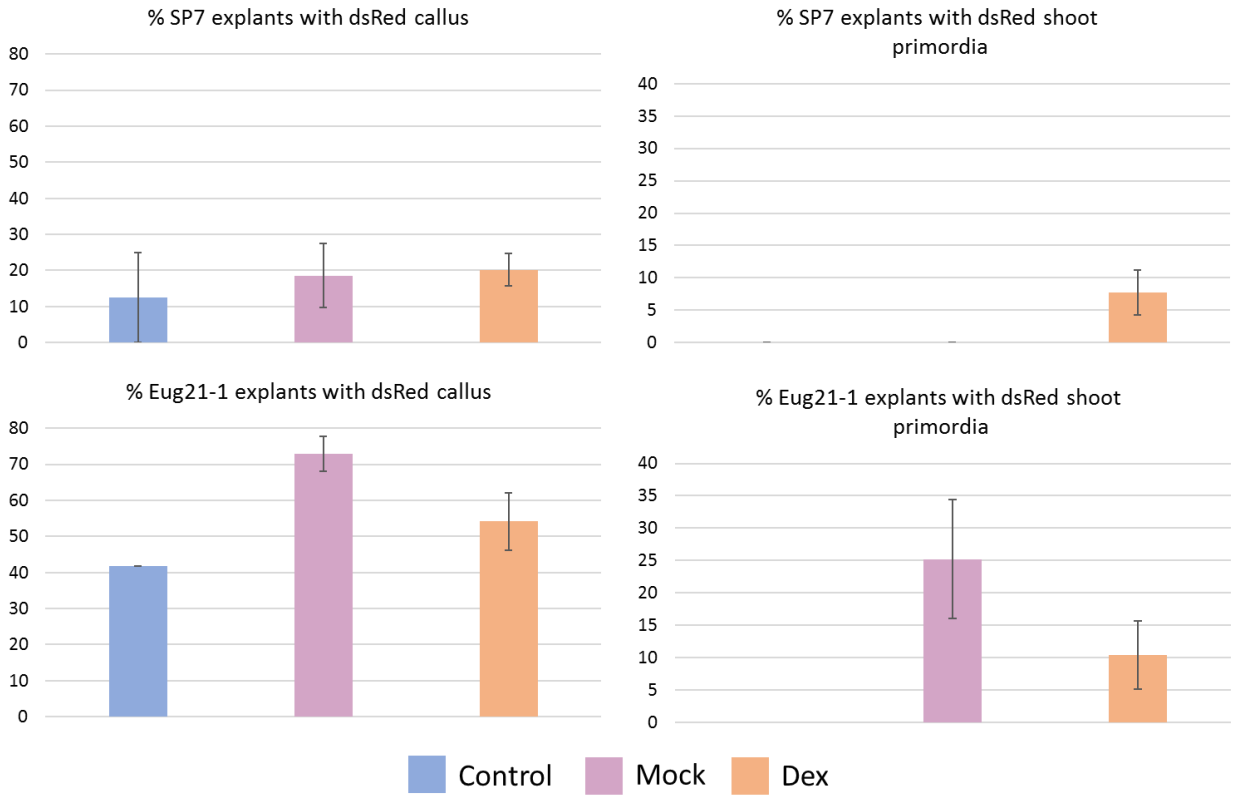


Figure D.7: Percentages of explants with dsRed callus and shoot primordia in two eucalypt genotypes during dexamethasone-inducible *Vitis* 4-mutation GR-GRF-GIF experiments. Error bars = standard error.

Genotype	No. independent replicate experiments	Construct	Number of explant	Number of plates (12 grid)	Avg. No. explant with transgenic callus/ plate	Avg. No. explant with TG shoot primordia /plate	% change vs control (callus)	P-value (callus)
'SP7'	1	Control	24	2	1.5	0	-	0.69
		Mock	42	4	1.5	0	+48.0%	
		Dex	52	5	2.2	0.8	+61.6%	
'Eug21-1'	1	Control	12	1*	5	0	-	0.15
		Mock	28	3	6.7	2.3	+74.8%	
		Dex	48	4	6.5	1.2	+30.0%	

Table D.1: Details and data from the *Vitis* 4-mutation GR-GRF-GIF experiments in two eucalypt clones. * = group had missing plates due to contamination on day 56 post-transformation. P-values calculated using Kruskal-Wallis non-parametric ANOVA.

Genotype	No. independent replicate experiments	Construct	Number of explant	Number of plates (12 grid)	Avg. No. explant with transgenic shoot(s)/ plate	Regen. Efficiency (total shoots/ total explants)	% change vs control	P-value
'717-1B4'	1	Control	86	8	3.6	33.7%	-	0.21
		Mock	55	5	4.8	43.6%	+29.4%	
		Dex	118	11	1.6	15.2%	-54.9%	
'353-53'	1	Control	62	6	3.5	33.8%	-	-
		Mock	84	7	3.1	26.2%	-22.5%	0.61
		Dex	94	8	0.0	0.0%	-100%	<0.01

Table D.2: Details and data from the *Vitis* 4-mutation GR-GRF-GIF experiments in two poplar clones. 717-1B4 regeneration rates did not significantly differ between groups (Kruskal-Wallis), while 353-53 regeneration rates were different among groups, and regeneration rates significantly differed ($P > 0.05$) between Control and Dex groups (pairwise Mann-Whitney test).