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Briefing
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Genetically Engineered American Chestnut:

Discussion of the performance limitations of Darling 58/54

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Introduction

The genetic modification (genetic engineering or GE) of trees is frequently suggested for increasing timber and pulp production, to mitigate climate change and provide carbon credits, as well as for restoration purposes, e.g. to protect trees from pests and diseases.

These projects are often accompanied by unrealistic claims; moreover, any such trees could pose high risks to forests and forest ecosystems.

A reliable long term risk assessment for the use of GE forest trees is near impossible. Risk assessments need to be able to ensure that no significant harm can come from the release and spread of a GE tree across time and space, in particular as a recall is likely to be impossible once reproduction and propagation has started.

So, what makes a risk assessment so challenging? What makes achieving a reliable trait so difficult, and how can side effects and knock-on effects be prevented?

The case of the genetically engineered American chestnut – named Darling 58 - illustrates very well some of the challenges of risk assessment and confirms the concerns raised around genetic engineering. Recent findings reported by The American Chestnut Foundation (TACF) confirm that the GE trees do not function as intended or predicted by their developers, nor is there an obvious way to move past this problem.

The American chestnut and fungal blight

Once a major canopy tree of the eastern forests of the USA and Southern Canada (Ontario), the American chestnut (*Castanea dentata*) was decimated and nearly wiped out by fungal blight. The fungus *Cryphonectria parasitica* is reported to have arrived in the US at the turn of the previous century with a nursery stock of Asian chestnuts, a species that is much less impacted by the fungus. Entering the tree through a wound, the fungus will spread with its hyphae. Their excretion of oxalic acid leads to cell death (necrosis) of tree tissue, resulting in deep cankers. Cell death will ultimately block the supply of water and nutrients to any part of the tree above the cankers, thus either killing the tree or reducing it to a bush-like existence, sprouting from the root collar or stump.

There are many ongoing projects that work without genetic engineering to establish blight-tolerant American chestnut trees, including selection of and breeding with partially blight-tolerant American chestnuts; crossing American chestnut with blight-tolerant Chinese chestnut followed by repeated back-crossing with American chestnut; and working with hypovirulence.

Plants have developed many forms and layers of defence that engage various networks of genes and feedback loops. Given time, they are well equipped to adapt and to form new ways to counter challenges, change responses, work with evolution. This capacity needs to be utilised in restoration efforts. As often pointed out by those working with forest trees – especially in restoration or sustainability projects – this work may span across the careers and lives of breeders, foresters, scientists, possibly for many generations.[1]

Genetically engineered American chestnut – Darling 58 and 54

Researchers at the State University of New York College of Environmental Science and Forestry (SUNY-ESF) are currently petitioning the US Department of Agriculture (USDA) for the deregulation of a genetically engineered 'blight-tolerant' American chestnut (Darling 58, now Darling 54) to serve as a "restoration" tree.

The researchers chose to genetically engineer American chestnut to produce OxO (oxalate oxidase), a compound that will break down and detoxify the fungal oxalic acid. To this end they made different 'gene constructs', combining the OxO gene from wheat with various genetic elements from other sources (Newhouse et al., 2014). A crucial component in any gene construct is the "promoter", a regulatory element in front of the gene that functions like an on/off switch. Promoters are commonly highly specific in their response to certain signals within the cell or organism. Thus, they can be, for example, development specific, tissue specific or task specific.

[1] Sara Fitzsimmons refers to it as working according to "tree time" in TACF Chestnut Chat 44: 15 Dec 2023 - Darling 58 & Science Strategy Update, Part 1. https://tacf.org/chestnut-chat/#darling-58-update-sept-15-2023

Deciding to use a switch that would be switched on all the time everywhere and at all developmental stages, the researchers chose the 35S CaMV promoter (from the cauliflower mosaic virus - CaMV), a type of promoter that is referred to as "constitutive" (as compared to "inducible"). As a constitutive promoter, 35S CaMV is outside the control of the plant's own regulatory system. In their petition for deregulation in 2020 the developers state:

> "*Lower levels of OxO expression via other promoters have been shown to result in low to intermediate blight tolerance in American chestnut (Section 10.5.1), so 35S is the most effective promoter tested to date for blight tolerance via OxO*." (Petition p.86)[2]

Whilst this 35S promoter is widely used in research, it can be unreliable and problematic in the long run, as repeatedly reported in the scientific literature – *see section* 'Not blight-tolerant or resistant', page 6.

To obtain trees that would contain the OxO gene, embryonic American chestnut tissue was transformed (genetically modified) using two different gene constructs linked together, one containing the 35S::OxO gene and the other containing a bacterial antibiotic resistance gene (NPTII)[3] from *Escherichia coli,* utilised here as a marker gene[4]. Using the bacterium *Agrobacterium tumefaciens* as a shuttle to get the constructs into the cells, the gene constructs will then integrate into the host's DNA at a random place in one or more chromosomes. As a result, different cells will have the linked constructs inserted in different places, in different copy numbers, or it may be that additional fragments of a construct are inserted. Each transformed cell will thus have a different genetic makeup. Any plant that manages to regrow via tissue culture from one of these cells will thus be the product of a single unique transformation event, each one named differently, such as D58 or D54.

What happened and what went wrong? (and what does it mean?)

Identity mix-up and failure to detect it

In late 2023, TACF discovered that there had been a major identity mix-up that resulted in years of work on the GE line D54 instead of the expected D58.

It is thought that the mix-up happened in the early stages of the project - possibly in 2016 - when D54 pollen samples were mistakenly labelled to be D58. Consequently, all offspring that researchers worked with were not D58 but D54.[5]

It is astonishing, however, that in all those years no one tested for the stability and integrity of the inserted transgene and its neighbouring DNA over the course of time, which should be a standard procedure. This would also have revealed the mix-up.

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[2] Newhouse et al. (2020) Petition for Determination of Nonregulated Status for Blight-tolerant Darling 58 American Chestnut (Castanea dentata), January 17. https://www.aphis.usda.gov/sites/default/files/19- 309-01p.pdf

[3] The NPTII (neomycin phosphotransferase) gene provides resistance to "(aminoglycoside) antibiotics such as kanamycin; neomycin; geneticin (G418), or paromomycin" and is equipped with a 'constitutive' promoter, in this case UBQ10 from the plant *Arabidopsis thaliana*. (Newhouse et al. 2020 – see footnote 2 - pages 85 & 87.

[4] Newhouse et al. (2020) - see footnote 2.

[5] TACF Briefing, Dec 2023; Darling 58/54 - Darling 54 FAQ: Why did TACF withdraw support from the Darling project? https://tacf.org/darling-58/

It is of serious concern that even the petition to the US Department of Agriculture for the deregulation of D58, submitted in January 2020, did not include tests that confirm the identity and integrity of the subject of that petition with regards to field trials and would not be providing genomic stability data. This should be seen as vital and paramount for trees intended for 'restoration' purposes.

Disruption of the tree's own gene by the OxO-transgene

It is commonly regarded as crucial to ensure that the integrity of the plant's own genes is preserved and not accidentally disrupted by the process of genetic engineering, for both performance and biosafety reasons. The most obvious case of disruption would be the insertion of a transgene into a plant's own gene and thus disabling the native gene.

This was exactly what happened in the case of Darling 54. Located on chromosome 4, the OxO gene construct got inserted into the sequence of a native gene called 'SAL1', thus disrupting it. The process of insertion also caused the deletion of 1069 nucleotides (base pairs) in 'SAL1'.[6] As a result, the native gene is no longer functional.

The consequences of this disruption are as yet completely unknown. Whilst the gene seems to have high similarities to the SAL1 gene of some other plants - where it is associated with drought and/or salinity tolerance - it has not been studied in chestnut.[7] Furthermore, "being involved" in specific traits does not mean a gene is not also involved in other traits and pathways. The loss of this gene's function may well impact the tree in other ways than expected, or perhaps weaken its stress response or alter its stress susceptibility. However, all of this is speculative and requires investigation.

High variability, even within sibling lines

Although it had been postulated that genetic engineering with the OxO gene would result in nothing else but the production of oxalate oxidase, and thus result in a higher level of resistance, this is clearly not the case according to the data provided by TACF. Whilst there are significant differences between non-transgenic and transgenic sibling lines, there is also a high degree of variability found amongst the GE trees, e.g. in growth rates, resistance or lack of resistance, canker size, and mortality. Reported by TACF, the following was found in field tests:

> *"...analysis indicated striking variability in Darling trees' blight tolerance, significant losses in growth competitiveness, reduction in overall fitness including stunted growth, leaf browning and curling, and increased mortality."*[8]

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[6] TACF Briefing. Darling 58/54 – Darling 54 FAQ. https://tacf.org/darling-58/

[7] Transgene position in Darling 54: Darling 54's transgene is located on Chromosome 4 within the sequence of a "Sal1 ortholog" (gene with high similarity to a gene known as Sal1 in other species). This gene has not been previously studied in chestnuts, so its function is hypothesized based on studies in other plants, where it has generally been linked to drought or salt tolerance. (SUNY-ESF 15 Dec 2023, https://www.esf.edu/chestnut/science-update/index.php#transgene)

[8] TACF Briefing. Darling 58/54 – Darling 54 FAQ

Stunted growth

Researchers at Purdue University conducted performance tests in nurseries as well as in the orchard and compared sibling trees that inherited the OxO construct (oxo+) with those that did not (oxo-). They found that there was a growth penalty of 15% for oxo+ trees in the first year (2021) and 25% in the second year (2022).[9]

This is a substantial growth penalty for the D54 trees, with a significant increase from year one to year two. Whilst it is not clear whether the growth lag might stabilise to around 25% per year, or increase or indeed decrease, it indicates clear competitive disadvantages for natural forest settings.

The reason behind this stunted growth has not been verified nor investigated, but a plausible candidate would be the presence of the 35S promoter with its constant activity. If the tree is pushed to produce oxalate oxidase in all tissues and all cells all the time, this will require energy and resources which will not be available for growth. Some refer to this as the "metabolic burden" of constitutive promoters and suggest the use of inducible promoters instead, "to function at certain stages of growth and development of an organism or a particular tissue which help in reduction of energy expenditure of the plant." (Kummari et al., 2020).

Other possibilities to investigate are the potential negative impact of the gene product itself (or products resulting from its activities) on cells and tissues where it is continuously produced(Amack & Antunes, 2020); or the possibility that the high activity of the 35S promoter enhances or triggers genes in its vicinity to become active, sometimes referred to as co-activation or leaked activation.

However, it may also be related to the effects of the process of genetic modification, such as the disruption of the 'SAL1' gene, the role of which is as of yet not understood but hypothesised to be part of a particular stress response, namely drought, and salinity. Another process-related candidate would be unintended small injuries to other genes due to the wider transformation process, which would not have been removed through back-crossing in this short time, especially if they happen to be located near the 35S::OxO gene. Such process-induced mutations have been repeatedly observed and detailed in publications e.g. (Latham et al., 2006; Wang et al., 2021). [10]

These unintended and unexplained phenomena require further investigation and research.

[9] Data from research at Purdue University, presented by Vasiliv Lakoba at TACF Webinar 15 Dec 2023.

[10] The insertion of the CRISPR/Cas transgene into grapevines using *Agrobacterium* and tissue culture were reported to have caused thousands of point mutations, with 230-377 of these in the coding region of genes. The researchers stated: " *... these variations were mainly produced by tissue culturing and/or* Agrobacterium *infection. Similar results have been reported in rice [17] and cotton [13]. These spontaneous mutations that occur in the exonic regions might affect the function of those genes,..."* (Wang et al., 2021)

Reduced survival and vigour

Darling offspring containing the 35S::OxO gene construct were observed to have a 65% survival rate as compared to the 95% for non-transgenic non-oxo siblings, where both were grown together under the same conditions in nurseries.[11]

Again, comparing oxo+ and oxo- siblings over a period of two years, it is reported that oxo+ trees (i.e. Darling 54) have significantly lower wellbeing and survival when under stress, such as resulting from an injury. With leaves starting to curl and to brown, this has been observed to lead from reduced photosynthesis and stunted growth to (at times) death.

Again, why there is reduced survival and vigour is so far unexplained and needs investigation. One could speculate that it is due to the interrupted 'SAL1' gene, as this seems to be related to the stress response network or system of the tree. However, it could instead be due to any of the candidates mentioned above or, of course, something completely different and not as yet hypothesised.

Not blight-tolerant or resistant

Following up on collaborative research, TACF reported that blight resistance was not holding up in D54 after inoculation with the fungus. In a 16-month experiment, cankers would initially be largely superficial, or a swelling occurred where the tree was attempting to block the fungus off by producing extra tissue. This mostly changed over time. Cankers were observed to become deep, with trees weakening and succumbing to blight, with only a few trees left in the mildly-affected category. Further observation is required. [12]

An important insight is the impossibility of making an accurate judgment based on initial findings – such as initial lab and greenhouse testing. There is the need, instead, to give sufficient time for developments to unfold.

These observations are in line with findings that investigated the rate of expression of the OxO gene over time and across 3 generations (founder, 1st and 2nd generations). Over time and over generations, increasing variability was observed, for example OxO gene expression in the stem of second-generation trees ranged from zero to much higher levels than the founder tree[13].

The working hypothesis as to why blight tolerance is so variable and why it is decreasing to zero in some trees is that the 35S::OxO genes are being silenced by the tree's own protection and defence mechanism.

Is that plausible? Very much so. In fact, this possibility should have been anticipated or predicted by the developers from the onset. The risk that transgenes under the control of the 35S promoter may be silenced has been known for a long time and cases have been

[11] Data presented by Vasiliv Lakoba at TACF Webinar Chestnut Chat 44: 15 Dec 2023 - Darling 58 & Science Strategy Update, Part 1. https://tacf.org/chestnut-chat/#darling-58-update-sept-15-2023 (https://www.youtube.com/watch?v=aGRW0VoIQUU)

[12] James R. McKenna reporting on experiments from Indiana., TACF Webinar Chestnut Chat 44: 15 Dec 2023 - Darling 58 & Science Strategy Update, Part 1. https://tacf.org/chestnut-chat/#darling-58-update-sept-15-2023

[13] Matthews (2020) - Https://experts.esf.edu/esploro/outputs/graduate/Oxalate-Oxidase-Quantification-and-Relative-Mrna/99871078504826#file – in Chestnut Chat: Darling 58 & Science Strategy Update part 1 https://www.youtube.com/watch?v=aGRW0VoIQUU https://www.youtube.com/watch?v=aGRW0VoIQUU&t=1884s

documented repeatedly, since first observed in 1990 in transgenic petunia[14] and followed by many others.[15]

The only defence plants have against an activity that is not under their control (such as an invading virus) is to shut down that activity by the methylation of specific DNA nucleotides, in this case, of the 35S promoter. This blocks the promoter (or, in other cases, sections of genes) and thus silences that gene, something which is heritable for generations.[16] It will however not show up in ordinary DNA sequencing, as it is not a change to the sequence of the DNA as such, but to its surface appearance.

Homozygosity for transgene is mostly lethal

According to Mendelian inheritance rules, crosses between D54 trees should result in a quarter (25%) of the offspring having two identical copies of the 35S::OxO gene, one from each parental tree, that is: being homozygous for this gene. However, this was not found to be the case. Using embryo rescue procedures, 2 out of 18 immature seeds were reported to be homozygous for OxO, carried out at SUNY-ESF. This ratio became still worse when taking chestnut burrs to full nut maturity and growing them into seedlings. Tested at Han Tan's lab at the University of Maine, only one seedling out of 39 was homozygous for OxO, that is 2.5% instead of 25%.[17] This indicates that homozygosity for 35S::OxO is mostly lethal or highly disadvantageous.

SUNY-ESF later reported having established 3 homozygous plants from 19 chestnuts, but it is not clear whether this was achieved via embryo rescue or done from mature seeds, nor whether the 18 immature seeds mentioned above were part of these 19.[18]

These are disturbing results, especially for the mature chestnut seeds, where only 2.5% instead of 25% were OxO/OxO homozygous. These results could be due either to the 35S::OxO construct and its activity and product, or to the mutational impact of the genetic engineering procedure, especially the disruption of the 'SAL1' gene. If the product of the 'SAL1' gene is crucial for any stage of development or for any stress-related situation, then higher lethality levels can be expected if both copies of the 'Sal 1' gene are dysfunctional. Of course, it may also be the result of an injury to a gene near the 'SAL1' insertion locus, that co-migrates with the construct.

All of this needs to be thoroughly investigated to fully understand the problems and risks of D54.

Furthermore, a genetically modified tree that reveals so many problems and has been so little investigated constitutes a clear risk and should not be grown where it could send its propagules – be it pollen or seed - into the wild.

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[14] Napoli et a. (1990)

[15] Mishiba et al. (2005) & Weinhold et al. (2013)

[16] E.g. Matsunaga et al. (2019).

[17] Reported by Tom Klak (UNE) at the TACF webinar Chestnut Chat: Darling 58 & Science Strategy Update part 1 – (at 43:20 minutes) https://www.youtube.com/watch?v=aGRW0VoIQUU

[18] ESF Darling Chestnut Science Summary - December 15, 2023. https://www.esf.edu/chestnut/science-update/index.php#homozygosity

The risks of GE forest trees and the limitations of risk assessment

Forest trees differ substantively from field crops yet are often treated as if the risks from their genetic modification are the same or can be assessed in the same way.

Annual crops and forest trees differ in at least 7 major ways that are relevant to risk assessment, including degree of domestication, life span, complexity of ecosystems, symbiotic partners and/or spatial distribution, and exposure to stressors. More detailed comparisons show that the knowledge gained from GE crops is insufficient to predict or analyse the risks posed by the release of GE trees.

> *"...forest trees may have more complex and less understood interactions with their environments than their agricultural counterparts. Many species are capable of dispersing large quantities of viable pollen to great distances and may cross with many sexually compatible individuals and species of naturally occurring plants. And unlike many agricultural crops, forest trees are genetically very diverse within a species, are long-lived, and are capable of persisting in unmanaged ecosystems."* (Finstad et al., 2007)

There is still a lack of understanding of the various levels and components relevant to risk assessment, their feedback loops and interactions, from the molecular level to population, ecosystem and biosphere level. This combined with the uncertainties around the effects of the genetic modifications on the tree across space and time, requires the application of the precautionary principle.

1 Trees have a low level of domestication

As genes interact and work together as part of the cellular, organism and environmental communication feedback system, it is to be expected that genetic modifications may have different effects or impacts depending on the genomic background and setting. While both performance and risk assessment of a crop plant that is genetically highly uniform can be regarded as valid across a whole population, trees, especially forest trees, have a low level of domestication and a rich genetic diversity and variability. The impacts of the outcrossing of intended or unintended modifications of a GE forest tree into this diverse natural setting cannot be predicted from the assessment of the initial population.

2 Trees have life spans of decades or centuries

Due to their long lifespans, trees are repeatedly exposed to a variety of stresses which can be divided into abiotic (cold, heat, drought, flood, fire, storms) and biotic (attacks by herbivores, pests, diseases). The ability to react to these, often extreme, stresses is linked to secondary metabolic pathways and requires a high degree of "*cross-talk and signalling processes between pathways*," much of which is not yet identified or fully understood (Isah, 2019).

Unintended alterations of any of the components of stress resistance may drastically alter the ability of the tree to respond to specific stresses. However, this impact may only become evident many years later, when these particular stresses happen to occur, and possibly only in future generations.

Furthermore, any negative effect on forest ecosystems caused by GE trees can become manifest at any point in time; can act over a very long period; and such effects can also be cumulative.

3 Pollen, seed and other reproductive plant materials are dispersed over long distances

Containment or the ability to recall should be seen as a major biosafety requirement if it cannot reliably be established that no harm can arise across time and space and across the breadth of variation and genetic diversity. The spread of transgenes into wider populations or related species is a national as well as transboundary biosafety (and prior informed consent) issue.

4 Trees have a large spatial distribution

Many trees are naturally present over a large geographical area and hybridisation is common. Whilst this is especially true for the genus *Populus,* chestnuts also have a wide range and are able to hybridise. This needs to be taken into consideration for risk assessment and decision-making, e.g.: what would be the impact (ecologically as well as legally) if the GE chestnut were to hybridise across its range?

5 Trees are an integral part of complex ecosystems (forests)

Field crops are constituents in tightly controlled cropping systems, with minimal interaction with other organisms (plant, animal, fungi or bacteria). Trees, however, are a major constituent of complex ecosystems (forests), which also provide ecosystem functions, habitats and food both to symbiotic partners, such as mycorrhizal fungi and associated species, and to animals and other plants in general. Unlike most agricultural plants, forest trees naturally persist and thrive in unmanaged ecosystems. This poses serious challenges for the risk assessment of GE forest trees.

For example, there are challenges when considering the potential impacts on soil. Will the modification affect interactions with soil organisms, especially micro-organisms, and if so, in which way? What are the consequences? What if the transgenes are transferred to soil-microorganisms by a mechanism called horizontal gene transfer? So far this question seems only to have been asked for agricultural systems, e.g. (Un Jan Contreras & Gardner, 2022), yet it might be particularly relevant for forest settings.

In the context of horizontal gene transfer, it should be highlighted that the CaMV 35S promoter as used in the Darling tree is not only active in plants. Transcription activity has been found in, for example, Atlantic salmon, frog eggs, some human cell lines and, importantly, "in bacterial cells, such as *Escherichia coli*, *Agrobacterium tumefaciens*, *Yersinia enterocolitica*, *Pseudomonas putida*, and *Acinetobacter*." (Amack & Antunes, 2020)

6 Trees are part of intricate communication systems

It is only in the last few decades that research has started to reveal that trees do not function as isolated individual organisms but are capable of supporting and communicating with other trees and functioning in a cooperative manner, both above as well as below ground. Because much of this activity appears to take place underground, it has long escaped the observation - and imagination - of forest scientists.

7 Trees affect water and climate systems

It is well established, for example, that trees, and in particular forests, are an integral part of water cycles and can act as climate buffers. Ensuring that genetically engineered forest trees do not undermine the integrity or resilience of vital forest ecosystems should be a significant biosafety consideration.

Concluding remarks

It is impossible to come to any final conclusions regarding the reasons for the failure of GE chestnut as so much data as well as knowledge is missing.

For example, it is not clear from any of the available materials:

whether or not fragments or partial copies of either of the two constructs used have accidentally inserted into the D54 genome;

whether the construct with the antibiotic marker gene is present and where it has inserted;

whether the wider area around the insertion site (the section that would be co-inherited with the OxO gene) has been thoroughly assessed for unintended modifications (transformation induced mutations);

The wider scientific community, including the risk research and risk assessment community, as well as the conservation community, will greatly benefit from a peer reviewed publication on the performance limitations observed with Darling 54, as well as detailed molecular information, including epigenetic data regarding gene silencing.

It has become evident once more that short term observations and simple greenhouse trials are insufficient to judge or predict the behaviour or performance of a GE tree. Any assumptions and claims should be taken with great caution if they are only based on standard greenhouse trials and if they are not based on long-term observations, e.g. decades, and several generations.

It would also be important to understand why the 35S promoter was not questioned earlier, as it has been known for many years to be a likely candidate for gene silencing.

This case of the failed attempt to achieve blight-tolerance in American chestnut via genetic engineering illustrates some of the many significant challenges with using genetic engineering and should be instructive as to the inability of GE to provide a quick solution to conservation or restoration needs.

Resources

ESF Darling Chestnut Science Summary. *December 15, 2023*.
https://www.esf.edu/chestnut/science-update/index.php

TACF Darling Performance Results. December 8, 2023. https://tacf.org/darling-58-performance/

References that are not detailed in the footnotes

Amack, S. C., & Antunes, M. S. (2020). CaMV35S promoter – A plant biology and biotechnology workhorse in the era of synthetic biology. *Current Plant Biology, 24*, 100179. doi:https://doi.org/10.1016/j.cpb.2020.100179

Finstad, K., Bonfils, A. C., Shearer, W., & Macdonald, P. (2007). Trees with novel traits in Canada: regulations and related scientific issues. *Tree Genetics & Genomes, 3*(2), 135-139. doi:10.1007/s11295-006-0080-y

Isah, T. (2019). Stress and defense responses in plant secondary metabolites production. *Biol Res, 52*(1), 39. doi:10.1186/s40659-019-0246-3

Kummari, D., Kummari, D., Palakolanu, S. R., Kishor, P. B. K., Bhatnagar-Mathur, P., Singam, P., . . . Sharma, K. K. K. (2020). An update and perspectives on the use of promoters in plant genetic engineering. *Journal of Biosciences, 45*, 1-24.

Latham, J. R., Wilson, A. K., & Steinbrecher, R. A. (2006). The mutational consequences of plant transformation. *J Biomed Biotechnol, 2006*(2), 25376. doi:10.1155/jbb/2006/25376

Matsunaga, W., Shimura, H., Shirakawa, S., Isoda, R., Inukai, T., Matsumura, T., & Masuta, C. (2019). Transcriptional silencing of 35S driven-transgene is differentially determined depending on promoter methylation heterogeneity at specific cytosines in both plus- and minus-sense strands. *BMC plant biology*, *19*(1), 24. doi:10.1186/s12870-019-1628-y

Mishiba, K., Nishihara, M., Nakatsuka, T., Abe, Y., Hirano, H., Yokoi, T., Kikuchi, A., & Yamamura, S. (2005). Consistent transcriptional silencing of 35S-driven transgenes in gentian. *The Plant Journal: for cell and molecular biology*, *44*(4), 541–556. doi:10.1111/j.1365-313X.2005.02556.x

Napoli, C., Lemieux, C., Jorgensen, R. (1990). Introduction of a chimeric chalcone synthase gene into Petunia results in reversible Co-suppression of homologous genes in trans. *Plant Cell*, 2, 279-289. doi:10.1105/tpc.2.4.279

Newhouse, A. E., Polin-McGuigan, L. D., Baier, K. A., Valletta, K. E. R., Rottmann, W. H., Tschaplinski, T. J., . . . Powell, W. A. (2014). Transgenic American chestnuts show enhanced blight resistance and transmit the trait to T1 progeny. *Plant Science, 228*, 88-97. doi:10.1016/j.plantsci.2014.04.004

Un Jan Contreras, S., & Gardner, C. M. (2022). Environmental fate and behaviour of antibiotic resistance genes and small interference RNAs released from genetically modified crops. *J Appl Microbiol, 133*(5), 2877-2892. doi:10.1111/jam.15741

Wang, X. H., Tu, M. X., Wang, Y., Yin, W. C., Zhang, Y., Wu, H. S., . . . Wang, X. P. (2021). Whole-genome sequencing reveals rare off-target mutations in CRISPR/Cas9-edited grapevine. *Horticulture Research, 8*(1), 11. doi:10.1038/s41438-021-00549-4

Weinhold, A., Kallenbach, M. & Baldwin, I.T. (2013). Progressive 35S promoter methylation increases rapidly during vegetative development in transgenic *Nicotiana attenuata* plants. *BMC Plant Biol* 13, 99. doi:10.1186/1471-2229-13-99