

From Hypothetical Risks to Proven Solutions:

The Scientific Case for Updating FSC Policy on GM Trees

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Executive Summary

Background: In 2000, the Forest Stewardship Council (FSC) published a policy guideline[1] on the potential risks associated with genetically modified (GM) trees, based on selected scientific opinion at that time.

The FSC interpretation emphasized a conservative precautionary approach: *Although GMOs may offer economic and environmental advantages, such as increased productivity, improved resistance to pests or harsh environmental conditions, and reduced reliance on pesticides, the FSC stresses that the uncertainties and possible negative impacts on biodiversity, ecosystem dynamics, and gene flow remain substantial.* These concerns were understandable in the early 2000s, when scientific knowledge on long-term ecological and health effects of GMOs was still limited, and global regulatory frameworks were evolving.

Since then, more than 25 years of extensive research, field trials, commercial plantings, and comprehensive risk assessments have generated a substantial body of evidence on GM tree safety[2].

Key Findings: ***None of the potential hazards listed in the FSC's 2000 interpretation has been scientifically validated or demonstrated to occur in real-world conditions.***

The accumulated evidence demonstrates that GM technologies, when properly developed and managed, are safe and do not cause adverse environmental impacts, but conversely have the potential to add environmental, social and economic benefits.

Some of the discussed traits theoretically can also be achieved through classical tree breeding without the use of GMOs or genome editing. The FSC approves these "risks" as long as they are not achieved through genetic engineering. This suggests a biased agenda against GM technology rather than its factual outcomes.

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[1] FSC-POL-30-602, FSC Interpretation on GMOs (Genetically Modified Organisms), Policy (POL), V(1-0). https://connect.fsc.org/document-centre/documents/resource/156

[2] James, D. (2024). Impact Assessment of Genetically Engineered Trees: An Overview on Risk Assessment and Management. In: Thomas T, D., Razdan, M.K., Kumar, A. (eds) Biotechnological Approaches for Sustaining Forest Trees and Their Products. Springer, Singapore. https://doi.org/10.1007/978-981-97-4363-6_16

Climate Change Urgency: Recent studies project productivity losses of 3-40% for eucalyptus plantations across Brazil due to climate change, with water deficits and temperature stress as primary drivers. GM trees offer adaptation solutions (drought tolerance, pest resistance) that cannot be developed quickly enough through conventional breeding to meet the pace of climate change[3].

Purpose of this document: This document provides updated, published scientific evidence, the vast majority from independent sources and all peer reviewed to inform FSC's ongoing policy development process, allowing members to make informed decisions based on current scientific knowledge.

By doing so, we can ensure that innovation is aligned with sustainability principles, while maintaining effective risk mitigation measures based on current scientific knowledge.

[3] Hn Palma J, Hakamada R, Moreira GG, Nobre S, Rodriguez LCE. (2021). Using 3PG to assess climate change impacts on management plan optimization of Eucalyptus plantations. A case study in Southern Brazil. Sci Rep. Feb 1;11(1):2708. doi: 10.1038/s41598-021-81907-z. https://pmc.ncbi.nlm.nih.gov/articles/PMC7851159/

Introduction: A Quarter Century of Evidence

In 2000, the Forest Stewardship Council (FSC) published FSC-POL-30-602 outlining potential hazards associated with genetically modified (GM) trees. This interpretation was based on scientific literature available at that time, largely from 1999 and earlier, representing the early years of agricultural biotechnology. At that time, concerns about GM crops were largely theoretical, with limited long-term field data available.

Since 2000, the scientific landscape has changed dramatically:

- Over 700 field trials with GM trees have been conducted globally as of 2010[4] with significant additional trials completed since then. Häggman et al.[5] established the international framework for environmental risk assessment of GM trees in plantation forestry, building on this extensive trial base. The methodology defined in this landmark *Plant Biotechnology Journal* paper has guided subsequent research and regulatory assessments.
- Extensive long-term evaluations have been conducted across the Northern Hemisphere: Europe has completed over 30 trials spanning seven nations[6], complementing US research that monitored over 20,000 transgenic trees over 20 years; both datasets confirm distinct stability and zero adverse ecological impacts[7].
- In Brazil, 11 approvals for GM eucalyptus events have been granted by CTNBio following extensive biosafety assessments. CQB (Certificate of Quality in Biosafety) areas cover approximately 412 hectares where trials are conducted with GM trees prior to their commercial release. All trials are subject to strict regulatory approval through the LPMA (Liberação Planejada no Meio Ambiente - permit for planned field release) submission to the CTNBio (the National

[4] Walter, C., Fladung, M. & Boerjan, W. (2010). The 20-year environmental safety record of GM trees. Nat. Biotechnol. 28, 656–658. https://doi.org/10.1038/nbt0710-656

[5] Häggman, H. et al. (2013). Genetically engineered trees for plantation forests: key considerations for environmental risk assessment. Plant Biotechnology Journal, 11(7), 785-798

[6] Pilate, G. et al. (2016). Lessons from 25 Years of GM Tree Field Trials in Europe and Prospects for the Future. In: Vettori, C., et al. Biosafety of Forest Transgenic Trees. Forestry Sciences, vol 82. Springer, Dordrecht. https://doi.org/10.1007/978-94-017-7531-1_4

[7] Strauss, S.H., Ma, C., Ault, K., Klocko, A.L. (2016). Lessons from Two Decades of Field Trials with Genetically Modified Trees in the USA: Biology and Regulatory Compliance. In: Vettori, C., et al. Biosafety of Forest Transgenic Trees. Forestry Sciences, vol 82. Springer, Dordrecht. https://doi.org/10.1007/978-94-017-7531-1_5

Technical Biosafety Commission of the Brazilian Government) and span approximately 18 years (2006–2024), from first planting to final harvest.

A note on what is being regulated. FSC's 2000 interpretation does not distinguish between two separate aspects of genetic engineering: the genetic engineering technology itself, and the trait outcome it produces. The distinction matters. For certain traits, such as disease and pest resistance, and within certain limits, lignin modifications, both classical breeding and genetic engineering theoretically can achieve the same parallel outcome. Ironically, FSC accepts the trait when produced by breeding while rejecting the identical trait when produced by GM. For other traits such as real climate-resilience traits, no breeding pathway is available, and GM is the only route. A prohibition on GM therefore does not merely slow innovation; for these traits it removes the outcome from the available toolbox entirely. The risk should be specifically assessed for trait and genes used, not the general methodology. The risks associated with the genetic engineering method are comparable with those associated with classical breeding [5, 8].

Overview of Genetically Modified Trees

Genetically modified (GM) trees are often discussed in relation to a set of potential environmental, biological, and socio-economic risks, as objections to their use.

Extensive scientific research, field trials, and regulatory assessments have generated robust evidence demonstrating that these risks are limited, well understood, manageable, and, when properly regulated, do not compromise environmental or human safety. Therefore, it is essential to examine each potential risk individually, based on scientific evidence and real-world experience.

Scientific Evidence: Addressing FSC's 2000 Risk Categories

The principal objections raised regarding the use of genetically modified (GM) trees can be grouped into five main categories. For each category below, we present:

(1) FSC's original concern,

(2) Accumulated scientific evidence from 25 years of research, and

(3) Conclusions and current risk assessment based on field data.

[8] Stacy D. Singer, John D. Laurie, Andriy Bilichak, Santosh Kumar & Jaswinder Singh (2021) Genetic Variation and Unintended Risk in the Context of Old and New Breeding Techniques, Critical Reviews in Plant Sciences, 40:1, 68-108, DOI: 10.1080/07352689.2021.1883826

1. Gene Flow and Invasiveness

Points 2, 3, 6, & 9, in the FSC risk policy page 5.

1.1. Original concerns related to this topic:

a. **Transgene dispersal to wild** or weedy populations, potentially leading to negative ecological impacts. These concerns are primarily associated with non-sterile GM trees or with trees exhibiting incomplete or unstable sterility mechanisms.

b. **Spread of herbicide resistance genes** through sexual reproduction, potentially resulting in the establishment of resistant trees in environments where they are undesirable, increased resistance in weed populations to the target herbicide, and/or increased herbicide use driven by the adoption of herbicide-tolerant GM trees.

c. **Asexual or horizontal gene transfer**, particularly the theoretical transfer of antibiotic resistance genes from GM trees to pathogenic microorganisms, as well as potential suppression of mycorrhizal fungi and other beneficial soil microorganisms.

d. **Reduced adaptability to environmental stress**, including unintended changes in interactions with other organisms and increased weediness or invasiveness arising from the introduction of novel traits.

1.2. Current Accumulated Scientific Evidence

- **Pollen Dispersal is Naturally Limited**: *Eucalyptus* exhibits limited pollen dispersal and relies on short-distance, non-wind-mediated pollination mechanisms[9]. Studies in eucalyptus show pollen dispersal follows a steep distance decay curve. In Brazilian conditions, effective pollination occurs primarily within 100 meters of the pollen source, declining rapidly to <2% by 200-300 meters[10;11]. No evidence of adverse environmental impacts related to gene flow or invasiveness has been

[9] da Silva, P. H. (2021). Gene flow and spontaneous seedling establishment around genetically modified eucalypt plantations. *New Forests, 52(3), 349-361*. doi:org/10.1007/s11056-020-09800-7

[10] da Silva, P.H.M., Sebbenn, A. M., Grattapaglia, D. (2015). Pollen-mediated gene flow across fragmented clonal stands of hybrid eucalypts in an exotic environment, Forest Ecology and Management, Volume 356, Pages 293-298, ISSN 0378-1127, https://doi.org/10.1016/j.foreco.2014.12.005

[11] da Silva, P.H.M., Abrahão, O.S. (2021). Gene flow and spontaneous seedling establishment around genetically modified eucalypt plantations. New Forests 52, 349–361. https://doi.org/10.1007/s11056-020-09800-7

documented over the past 28 years of research, field trials, and commercial use of GM crops and trees[12; 13; 14].

- **Eucalyptus is Non-Invasive**: In Brazil, where eucalyptus has been planted for over 100 years, *Eucalyptus* species remain exotic but non-invasive. Despite millions of hectares of plantations, eucalyptus does not establish self-sustaining populations outside managed plantations[15].
- **No Horizontal Gene Transfer Detected:** There is no scientific evidence supporting the occurrence of horizontal gene transfer from GM plants to microorganisms under natural conditions[16;17]. This theoretical risk, while addressable through regulatory review, has not materialized in practice.
- **Currently Deployed GM Traits Do Not Enhance Fitness**: The traits already introduced into planted eucalyptus trees through genetic modification do not possess ecological advantages that would enhance survival or competitiveness outside plantation environments[9;18;19]. Furthermore, all the suggested traits in Section 1.1d above, can theoretically be achieved through classical tree breeding without the use of GMOs or genome editing. The FSC approves this "risk" as long as it is not achieved through genetic engineering.

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[12] Goodman, R. (2024). Twenty-eight years of GM food and feed without harm: why not accept them? *GM Crops & Food, 15(1):40–50.* doi:org/10.1080/21645698.2024.2305944

[13] James, D. (2024). Impact Assessment of Genetically Engineered Trees: An Overview on Risk Assessment and Management. *Biotechnological Approaches for Sustaining Forest Trees and Their Products (pp. 425–462). Springer.* doi:org/10.1007/978-981-97-4363-6_16

[14] Pinheiro, A. d. (2023). Five-years post commercial approval monitoring of eucalyptus H421. *Frontiers in Bioengineering and Biotechnology, 11:1257576*. doi:org/10.3389/fbioe.2023.1257576

[15] da Silva, P. H. (2021). Gene flow and spontaneous seedling establishment around genetically modified eucalypt plantations. *New Forests, 52(3), 349-361*. doi:org/10.1007/s11056-020-09800-7

[16] Philips, J. G. (2022). Horizontal gene transfer from genetically modified plants. *Frontiers in Bioengineering and Biotechnology, 10: 971402*. doi:org/10.3389/fbioe.2022.971402

[17] European Food Safety Authority (EFSA), M. A.-B. (2025). Assessment of the 2023 post-market environmental monitoring report on the cultivation of genetically modified maize MON 810 in the EU. EFSA Journal, 23(8), e9613. doi:org/10.2903/j.efsa.2025.9613

[18] Pinheiro, A. d. (2023). Five-years post commercial approval monitoring of eucalyptus H421. *Frontiers in Bioengineering and Biotechnology, 11:1257576*. doi:org/10.3389/fbioe.2023.1257576

[19] European Food Safety Authority (EFSA), M. A.-B. (2025). Assessment of the 2023 post-market environmental monitoring report on the cultivation of genetically modified maize MON 810 in the EU. EFSA Journal, 23(8), e9613. doi:org/10.2903/j.efsa.2025.9613

1.3. Conclusions and current risk assessment

One must distinguish between the spread of the GM tree event itself to the environment, which has the same low likelihood as for any cultivated crop or tree, particularly eucalyptus in Brazil, and gene flow through crossing into a wild population. Both gene flow risks are well-characterized and are manageable through standard silvicultural practices. The international scientific community has established clear assessment points, experimental designs, and monitoring protocols[20] and extensive field evidence shows no adverse impacts over 25+ years of monitoring. Eucalyptus-specific studies in Brazil demonstrate effective pollination occurs primarily within 100m, with gene flow declining to <2% by 200-300m[21;229]. Risk mitigation is straightforward. Established risk mitigation practices, including physical isolation distances, border rows, monitoring protocols and refuge areas, are effective and routinely applied in operational tree farms[23].

Furthermore, the FSC does not address the risk of gene flow originating from non-GM eucalyptus hybrid crosses, even in cases where the resulting progeny is significantly different from the parents and could potentially lead to more harmful outcomes. The FSC appears to approve this "risk" as long as no GM technology is involved.

[20] Häggman, H. et al. (2013). Genetically engineered trees for plantation forests: key considerations for environmental risk assessment. Plant Biotechnology Journal, 11(7), 785-798

[21] da Silva, P.H.M., Sebbenn, A. M., Grattapaglia, D. (2015). Pollen-mediated gene flow across fragmented clonal stands of hybrid eucalypts in an exotic environment, Forest Ecology and Management, Volume 356, Pages 293-298, ISSN 0378-1127, https://doi.org/10.1016/j.foreco.2014.12.005

[22] da Silva, P.H.M., Abrahão, O.S. (2021). Gene flow and spontaneous seedling establishment around genetically modified eucalypt plantations. New Forests 52, 349–361. https://doi.org/10.1007/s11056-020-09800-7

[23] James, D. (2024). Impact Assessment of Genetically Engineered Trees: An Overview on Risk Assessment and Management. *Biotechnological Approaches for Sustaining Forest Trees and Their Products (pp. 425–462). Springer.* doi:org/10.1007/978-981-97-4363-6_16

2. Biodiversity in Planted Tree Areas

Point 1, in the FSC risk policy page 5.

2.1. Original concerns related to this topic:

a. Reduction in genetic and landscape-level diversity, based on the concern that plantations established with one or a few transgenic clones may exhibit lower diversity compared to plantations using species or varieties derived from conventional tree breeding.

2.2. Current Accumulated Scientific Evidence

- Landscape-level biodiversity in plantations is primarily determined by silvicultural practices and land-use planning, rather than by the transgenic nature of planting material. This is a general agricultural/plantation issue - the use of GM trees does not inherently reduce biodiversity, as the GM trait introgression is applied to all available breeding varieties.

2.3. Conclusions and current risk assessment

Eucalyptus tree farm biodiversity is determined by silvicultural practices and conventional tree breeding rather than any specific GM trait.

3. Effects on Target and Non-Target Organisms

Points 4 & 8, in the FSC risk policy page 5.

3.1. Original concerns related to this topic:

a. Increased resistance in target insect pests, as well as potential deleterious effects on natural enemies of target pests and on non-target organisms, such as pollinators, butterflies, and soil microorganisms, associated with the use of insect-resistant GM trees.

b. Reduced biodiversity of organisms dependent on flowers and fruits, potentially resulting from the use of sterile GM trees.

3.2. Current Accumulated Scientific Evidence

- Pest resistance is a significant target of classical tree breeding as well. Although this approach is more limited, it theoretically can be achieved without the use of GMOs or genome editing. The FSC approves this “risk” as long as it is not achieved through genetic engineering.

- Eucalyptus trees are naturally resistant to many insects, as all plants are. The cultivation of Eucalyptus outside its natural range had much more impact on the natural ecosystem than introducing a single insect resistance trait.
- Well-established Insect Resistance Management (IRM) strategies are implemented to prevent or delay the development of resistance in target pest populations[24].
- Insect-resistance GM technologies are highly target-specific and have been shown to be safer and more environmentally benign than conventional broad-spectrum chemical pest control[25].
- No sterile GM tree technologies are currently deployed in commercial forestry.

3.3. Conclusions and current risk assessment

Since most tree farms are managed by centralized bodies rather than private farmers, the compliance to IRM regimes is high, resulting in low resistance development compared to other pest control methods.

The high specificity of GM plants using Bt pesticidal proteins and RNAi technologies makes these methodologies amongst the safest pest control methods available with potential to increase biodiversity in their areas of deployment[26].

Extensive safety trials have approved the well-being of flowers and pollinators.

[24] Tabashnik, B. E. (2023). Global patterns of insect resistance to transgenic Bt crops: the first 25 years. *Journal of Economic Entomology, 116(2), 297-309*. doi:org/10.1093/jee/toac183

[25] Meissle, M. N. (2022). Does the growing of Bt maize change abundance or ecological function of non-target animals compared to the growing of non-GM maize? A systematic review. *Environmental evidence, 11(1), 21*. doi:doi.org/10.1186/s13750-022-00272-0

[26] Janet E. Carpenter (2011) Impact of GM crops on biodiversity, GM Crops,2:1, 7-23, DOI: 10.4161/gmcr.2.1.15086 https://doi.org/10.4161/gmcr.2.1.15086

4. Specific Traits

Points 5, in the FSC risk policy page 5.

4.1. Original concerns related to this topic:

a. Alterations in structural integrity, adaptation, and pest resistance, as well as changes in wood decay rates, soil structure, soil biology, or soil fertility, particularly in GM trees with modified lignin composition.

4.2. Current Accumulated Scientific Evidence

- Potential environmental effects associated with specific traits, such as modified lignin content, are systematically assessed as part of comprehensive GMO environmental risk assessments[27;28].
- The trait of concern, such as pest resistance, and lignin modification which can increase wood decay, are major targets of classical tree breeding. These traits theoretically can be achieved without the use of GMOs or genome editing. The FSC approves this "risk" as long as it is not achieved through genetic engineering. Relying exclusively on conventional breeding may limit the speed, precision, or scope of achievable outcomes when compared to an integrated approach.
- FSC lists "changes in wood decay rates, soil structure, and soil biology" as concerns but does not name a specific harm. The same shifts happen routinely every time one eucalyptus clone is replaced with another in commercial farms, with non-GM clones, without FSC objection.
- Switching from one tree species to another, for example planting eucalyptus where pine grew before, changes soil quality, biology and decay rates more than substituting one eucalyptus clone with a GM eucalyptus clone[29]. FSC accepts

[27] James, D. (2024). Impact Assessment of Genetically Engineered Trees: An Overview on Risk Assessment and Management. *Biotechnological Approaches for Sustaining Forest Trees and Their Products (pp. 425–462). Springer.* doi:org/10.1007/978-981-97-4363-6_16

[28] Avisar, D. D. (2023). Safety of genetically modified glyphosate-tolerant eucalyptus designed for integrated weed management. *Advances in Weed Science, 41*. doi:org/10.51694/AdvWeedSci/2023;41:00019

[29] Zhang, K., Zheng, H., Chen, F. L., Ouyang, Z. Y., Wang, Y., Wu, Y. F., ... & Xiang, X. W. (2015). Changes in soil quality after converting Pinus to Eucalyptus plantations in southern China. Solid Earth, 6(1), 115-123

species-level changes routinely under FSC-STD-01-001 V5-2 [30], which permits the use of exotic species like eucalyptus where they provide clear and substantial benefits and are managed to prevent invasive spread. The clone-level change at issue which is the subject of this objection is significantly smaller from a biological perspective.

4.3. Conclusions and current risk assessment

All the suggested concerns have been and are continuing to be rigorously verified through extensive field and lab safety trials. Furthermore, the FSC policy permits similar suggested risks for the same traits additions that are affected by classical breeding, which suggests a biased agenda against the technology rather than its outcomes. It should be stressed that all the technology changes are effected in a closed, contained laboratory environment and the technology per se, has no ability to effect impact – only the actual product – the modified tree - should be at issue, thus emphasizing the value of risk analysis on a product-by-product (or trait-by-trait) basis, not a methodology analysis.

5. Social and Economic Concerns

Points 7, in the FSC risk policy page 5.

5.1. Original concerns related to this topic:

a. Restricted or monopolistic access to benefits arising from the high costs of development or limited availability of GM tree technologies, potentially disadvantaging small producers and local communities.

5.2. Current Accumulated Scientific Evidence

- The benefits of GM tree technologies are increasingly shared with small outgrowers and across the entire plantation value chain[31].

[30] FOREST STEWARDSHIP COUNCIL (FSC). FSC-STD-01-001 V5-2 EN: FSC principles and criteria for forest stewardship. Bonn: FSC International Center, 2018. Disponível em: https://fsc.org/sites/default/files/2019-05/FSC-STD-01-001%20V5-2%20EN.pdf.

[31] Suzano. (2024). Genetically Modified Tree Policy, Políticas Corporativas PC.00.0044. *Transparency and shared benefits along the plantation value chain*. Retrieved from https://www.futuragene.com/wp-content/uploads/2024/08/Genetically-Modified-Trees-Policy-EN.pdf

- Full transparency is maintained throughout the development, regulatory approval, and deployment of GM trees[32;33].
- Human and animal safety are rigorously regulated under national and international biosafety frameworks[34].

5.3. Conclusions and current risk assessment

***The acknowledged benefits of GM technologies will be shared as we will share the best tree varieties (including ultimately genetically modified clones when approved for commercial planting)* <u>at no extra cost to our partner farmers</u>.**

GM tree technologies developed by companies like Suzano/FuturaGene include explicit commitments to benefit-sharing with farmers and local communities. Suzano's publicly available GM Tree Policy[35] establishes governance frameworks, advisory committees, and transparency mechanisms that address historical concerns about shared value.

[32] Avisar, D. D. (2023). Safety of genetically modified glyphosate-tolerant eucalyptus designed for integrated weed management. *Advances in Weed Science, 41*. doi:org/10.51694/AdvWeedSci/2023;41:00019

[33] Suzano. (2024). Genetically Modified Tree Policy, Políticas Corporativas PC.00.0044. *Transparency and shared benefits along the plantation value chain*. Retrieved from https://www.futuragene.com/wp-content/uploads/2024/08/Genetically-Modified-Trees-Policy-EN.pdf

[34] James, D. (2024). Impact Assessment of Genetically Engineered Trees: An Overview on Risk Assessment and Management. *Biotechnological Approaches for Sustaining Forest Trees and Their Products (pp. 425–462). Springer.* doi:org/10.1007/978-981-97-4363-6_16

[35] Suzano. (2024). Genetically Modified Tree Policy, Políticas Corporativas PC.00.0044. Transparency and shared benefits along the plantation value chain. Retrieved from https://www.futuragene.com/wp-content/uploads/2024/08/Genetically-Modified-Trees-Policy-EN.pdf

Conclusion

The accompanying scientific papers represent the consensus view of the international forest biotechnology research community, published in the world's leading peer-reviewed journals including *Nature Biotechnology*, *Plant Biotechnology Journal*, and *New Phytologist*. These papers document over 25 years (1988-present) and more than 700 field trials with GM trees across North America, Europe, Asia, and South America, conducted by research institutions including **VIB (Belgium), INRA (France), Oregon State University (USA), Thünen Institute (Germany)**, and dozens of other leading universities and research centers.

The authors include many of the world's most distinguished plant scientists: **Wout Boerjan (VIB/Ghent University, >30,000 citations, winner of the Marcus Wallenberg Prize), Steven H. Strauss (Oregon State University, leading forest geneticist), Christian Walter (formerly at Scion, New Zealand), and Gilles Pilate (INRA, France).** Collectively, these scientists have published hundreds of peer-reviewed papers and represent decades of field research experience with regulatory authorities worldwide.

The evidence is unequivocal: **no field trial over 25+ years has reported any substantive harm to biodiversity, human health, or the environment**. Non-target effects were consistently within the range of natural variation. Transgene stability, containment strategies, and soil microbial communities have been extensively documented. This body of evidence directly addresses and refutes the hypothetical risks that form the basis of FSC's current prohibition on GM trees.

We invite FSC to enter into constructive dialogue on the use of GM in forestry based on current scientific evidence, and to become a pioneer in developing the governance systems which would enable the use of critical technologies to address global sustainability imperatives.

Acknowledgments

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APPENDIX:

Scientific Peer-Reviewed Papers Booklet

Lessons from 25 Years of GM Tree Field Trials in Europe and Prospects for the Future

G. Pilate, I. Allona, W. Boerjan, A. Déjardin, M. Fladung, F. Gallardo, H. Häggman, S. Jansson, R. Van Acker and C. Halpin

Abstract It is common agronomic practice to perform a formal evaluation of the behaviour of new varieties under natural field conditions. Accordingly, shortly after the optimization of genetic engineering techniques on trees, a number of field trials were set up to assess GM trees modified for different genes. Here, we review the work that has been done in this arena in Europe over the last 25 years, and summarize what we learned from these experiments. GM tree field trials remain the exception rather than the rule in Europe. Several trials have been destroyed by anti-GMO activists and it is becoming increasingly difficult to obtain authorization for a GM tree field trial. These increasing constraints on GM tree trials within

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Europe are both surprising and counter-productive as we learned a lot from the past 25 years of experiments and the results were promisingly positive: (1) Phenotypic effects resulting from transgene expression in GM trees grown in the field appears to be stable, albeit variable; (2) most field studies have validated earlier observations made under greenhouse conditions, although in some cases the modification of target traits was less obvious in fluctuating field environments, and in a few cases had severe growth and developmental penalties; (3) non-target effects were consistently within the range of natural variation. Overall, the European GM tree field trials failed to exemplify any significant tangible risks. Based on this evidence, it seems appropriate that Europe should now move forward beyond small confined trials to larger scale experiments better fitted to a broader context of evaluation and environmental assessment.

1 Introduction

Forests are crucial natural resources for the earth ecosystem, as they provide a diversity of services including recycling carbon dioxide into oxygen, acting as a carbon sink, participating in climate regulation, protecting soil, and water and serving as a genetic reserve for biodiversity. For humankind, forests also serve as recreational areas, as a source of lumber, pulp, and paper, as a source of energy for cooking and heating, and now as a feedstock for bioenergy, biofuels and biomaterials. To respond to the growing demand for wood and alleviate pressure on natural forests, intensively managed forest plantations have been developed to increase productivity and shorten rotation time. Among other opportunities, GM technology is one potentially useful tool to create improved tree genotypes that enhance the efficiency and value of such plantations and of downstream wood uses.

GM technology was first developed more than 30 year ago, in the early 1980s: the first articles on GM tobacco transformed through *Agrobacterium tumefaciens*-mediated DNA transfer were published in 1983 (Barton et al. 1983; Herrera-Estrella et al. 1983). This transformation method was very rapidly transferred to other dicot plant species and in 1987 the first GM poplar tree was produced (Fillatti et al. 1987). Conifers, like monocots, proved to be more recalcitrant to *A. tumefaciens* infection and the first report of the production of a GM conifer by particle acceleration was not published until 1993 (Ellis et al. 1993). Taking advantage of the power of somatic embryogenesis in conifers, an efficient *A. tumefaciens* transformation procedure was eventually developed, first for larch (Levée et al. 1997) and later on for other conifer species. Today, GM materials can be produced for a wide array of tree species, even though this success is still often limited to a small number of genotypes within a given species.

Tree GM technology was first used in basic research studies to gain a better knowledge of biological mechanisms specific to these large, long-lived, perennial species. The evaluation of GM trees was initially mostly restricted to greenhouses.

Table 1 List of GM tree field trials planted in Europe from 1988-present and described in this chapter

Trial name and clones used	Promoter/transgene	Years	Area	Publications
BELGIUM				
Herbicide tolerance				
clone 064 (*P. deltoides* × *P. trichocarpa*) and clone 357 (*P. alba* × *P. tremula*)	*bar*	1988–1990 and 1991–1999	nd	De Block (1990)
Lignin modification				
717-1B4	35S::S*ccr*; 35S::AS*ccr*	2009–2017	331 m^2	Van Acker et al. (2014)
717-1B4	35S::RNAi*cad*	2014–2022	1311 m^2	Unpublished
FINLAND				
Pollination trials				
P. sylvestris, *P. abies*	35S::*uid*A	1996–2002		Aronen et al. (2003), Clapham et al. (2003)
Growth				
B. pendula	Nos::*npt*II; *lac*Z	1990–1991		Unpublished
B. pendula	35S::*Rbc*S; 35S::*Nr*; 35S::*npt*II	2000–2005 destruction		Unpublished
Tree interactions with fungi and insects				
B. pendula	35S::*chitinase* IV; 35S::*Bp4CL1*	2000–2003		Pasonen et al. (2005), Pasonen et al. (2009)
Sterility				
B. pendula	pMADS::*barnase*	2005–2012		Unpublished
T89, 51 (hybrid aspen)	pLMX5::*ERF*; *SLAC*1	2013–2018		Unpublished
FRANCE				
Herbicide tolerance				
Huneghem, Beaupré (*P. deltoides* × *P. trichocarpa*)	*bar*	1994–2002	8000 m^2	Unpublished results

(continued)

Table 1 (continued)

Trial name and clones used	Promoter/transgene	Years	Area	Publications
Stability and expression of foreign genes				
717-1B4 (*P. tremula* × *P. alba* [hybrid aspen])	35S::*uid*A	1991–1999	<100 m^2	Hawkins et al. (2003)
717-1B4	35S::*uid*A; pcad::*uid*A	1996–2003	1400 m^2	Hawkins et al. (2003)
Insect tolerance				
353-38 (*P. tremula* × *P. tremuloides* [hybrid aspen])	35S::*OC*I	1995–1999	945 m^2	Unpublished
Lignin modification				
717-1B4	35S::AS*cad*; 35S::AS*comt*	1995–2007	440 m^2	Lapierre et al. (1999), Pilate et al. (2002)
717-1B4	35S::AS*ccr*; 35S::AS*ccoamt*	1999–2007	925 m^2	Leplé et al. (2007)
717-1B4	35S::AS*cad*; 35S::AS*comt*; 35S::AS*ccr*; 35S::AS*ccoamt*	2007–2013	1365 m^2	Van Acker et al. (2014)
GERMANY				
Stability and expression of foreign genes				
Esch5 (*P. tremula* × *P. tremuloides* [hybrid aspen]), Brauna11, W52 (*P. tremula* [aspen])	35S::*rol*C; rbcS::*rolC*	1996–2001	1500 m^2	Fladung et al. (2004), Fladung and Hoenicka (2012)
Horizontal gene transfer				
T89, Esch5 (*P. tremula* × *P. tremuloides* [hybrid aspen])	GPD::*bar*	2000–2003	1000 m^2	Nehls et al. (2006)
Phytoremediation				
717-1B4	*Gsh*I	2002–2004	2500 m^2	Peuke and Rennenberg (2006)
717-1B4	*Gsh*I	2003–2005	2 × 2500 m^2	

(continued)

Table 1 (continued)

Trial name and clones used	Promoter/transgene	Years	Area	Publications
SPAIN				
Increased yield				
717-1B4	*gs*	nd	nd	Jing et al. (2004), El-Khatib et al. (2004), Pascual et al. (2008)
717-1B4	*CsRAV1*	2012-ongoing	204 m^2	Moreno-Cortés et al. (unpublished)
SWEDEN				
Stability and expression of foreign genes				
T89	13 different	1994	500 m^2	
Modification of photoprotection				
T89	PsbS RNAi	2004, 2007	2 m^2	Unpublished
Increased yield				
T89	16 different	2010-ongoing	1000 m^2	Unpublished
T89	7 different	2011-ongoing	500 m^2	Unpublished
T89	14 different	2012-ongoing	1000 m^2	Unpublished
Altered wood properties				
T89	7 different	2011-ongoing	500 m^2	Unpublished
T89	21 different	2014-ongoing	500 m^2	Unpublished
Altered phenology				
T89	27 different	2014-ongoing	1000 m^2	Unpublished
UNITED KINGDOM				
Lignin modification				
717-1B4	AS*cad*; AS*comt*	1996-destruction 1999		Pilate et al. (2002), Halpin et al. (2007)

Note Some other field trials with GM trees have been planted in Europe during this period, but data on these assays are not available

But field trials rapidly became necessary especially to take into account the effects of environmental variations on plant phenotype. This was particularly relevant for GM trees, as trees grown in the greenhouse quickly became cramped, with severely limited space for trunk, canopy and root development. Indeed, studies carried out in the greenhouse are necessarily restricted to very young trees (less than two-year-old) still at a juvenile stage. With the prospect of applied research to deploy economically interesting GM tree genotypes, it rapidly became necessary to evaluate GM trees in field tests, in just the same way that new non-GM genotypes bred through classical controlled crosses would be trialled.

Safety and containment procedures for such trials had to take several issues specific to trees into account (e.g. long lifetime, phase changes from juvenile to mature, bud dormancy, long-distance seed dispersal) in order to limit potential risks and accidental escape. The purpose of a rather high proportion of the first field trials with GM trees was precisely to assess what these potential risks might be. The first full field trials with GM trees were installed France in 1991 and a number of others were established in subsequent years throughout the nineties, in France, England, Belgium, Germany, Spain, Finland and Sweden (Table 1). At this time, there was already an administrative framework and a scientific evaluation procedure to control the establishment, maintenance and the final destruction of every field trial. With time, the conditions for obtaining authorization became more and more cumbersome, the risk of vandalism of trials increased, and the funding for studies involving GM plants became rarer. As a consequence, the years 2000–2010 were characterized by a decrease in the number of GM tree field trials in Europe, while the number was increasing in other parts of the world.

What did we learn from these experiments? This is the topic of this chapter which will review the different field tests performed in seven European countries over the last 25 years (in Belgium, Finland, France, Germany, Spain, Sweden and United Kingdom). Most European GM tree field trials for which published data is available are reported on in this paper.

2 Field Tests in Belgium (4 Field Trials with GM Poplar)

A first field trial with transgenic poplars was established in 1988 in Geraardsbergen, Belgium, with trees engineered for herbicide tolerance (De Block 1990). Three years later, in 1991, a second field trial with the same herbicide tolerant poplar lines was established (Report WIV-ISP 2010). Between then and 2002, more than 120 GM crop field trials including three on GM trees (apple, poplar) were conducted in Belgium, the majority by companies such as Plant Genetic Systems NV, Aventis Cropscience NV and Monsanto Europe. After 2002, the flourishing field trial culture in Belgium came to an end. The combination of uncertainty about what the implementation of the 2001/18 EU Directive would bring, and a number of field trial destructions, made companies decide to stop doing field trials (Custers 2009). This situation lasted until November 2007, when VIB requested permission for a field trial

with poplars (*Populus tremula* × *P. alba*) genetically modified in lignin biosynthesis with the purpose of using them for bioethanol production. Despite positive opinions from the Biosafety Advisory Council and the Flemish minister for environment, the federal ministers declined the field trial request, resulting in a political conflict (The Poplar Files, www.vib.be). Ultimately, VIB was forced to go to the Council of State to have the negative decision suspended. In December 2008, the Council of State ruled in favour of VIB and finally, in February 2009, VIB received permission to initiate a field trial with GM poplars for a period of 8 years. This permission can be considered a milestone in Belgian field trial history (Custers 2009).

The field trial in question (Fig. 1) was planted in May 2009 and contained two transgenic lines (FS3 and FS40) and a control wild type line (all in *P. tremula* × *P. alba*). The field itself consisted of six randomized blocks for each line, each block containing 20 clonally propagated trees. The transgenic trees were down-regulated in the gene encoding cinnamoyl-coenzymeA reductase (CCR), an enzyme involved in the biosynthesis of lignin. These GM poplars were made in the framework of the EU-funded project TIMBER. Downregulation of CCR typically results in reduced lignin content and an orange-wine red coloration of the xylem upon debarking (Leplé et al. 2007). As lignin is considered as the most important limiting factor in the conversion of plant biomass into fermentable sugars

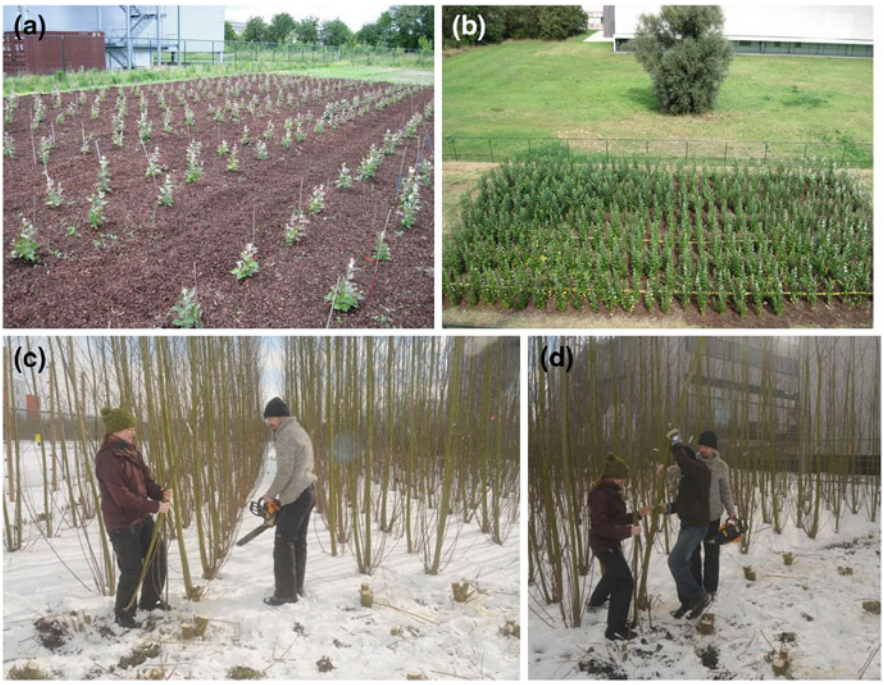

Fig. 1 Belgium CCR field trial. **a** 2 weeks after planting in May 2009. **b** 20 August 2009: after 3 months of growth. **c** March 2013: harvest

(Chen and Dixon 2007; Van Acker et al. 2013), the aim of this field trial was to investigate whether wood from field-grown lignin-deficient poplars was easier to process into bioethanol, compared to wild type poplars. The study demonstrated a large variation in the level of downregulation in individual stems, reflected by a variation in the intensity and pattern of red xylem coloration (from fully white stems, through patchy red coloured stems, to completely red stems). This variability in red coloration allowed the researchers to demonstrate a correlation between the level of CCR downregulation and the efficiency of wood processing into fermentable sugars (saccharification) and ethanol. However, strong downregulation of CCR also affected biomass yield, which outweighed the improved bioethanol production in most lines, a problem that probably could be solved by targeting the downregulation of lignin genes specifically to the fibres (Van Acker et al. 2014).

After the difficulties in obtaining regulatory permission for the establishment of a field trial with the CCR down-regulated poplars, a new era for field trial experiments in Belgium had started. In 2011, a two-year field trial with GM potato lines with improved resistance to *Phytophthora infestans* was established, while in 2012, permission was granted for a field trial with GM maize to demonstrate altered growth characteristics. In 2013, VIB obtained permission, for a period of 8 years, for the fourth GM poplar field trial in Belgian history (GMO register European Commission).

In this most recent poplar field trial, *P. tremula* × *P. alba* is down-regulated in another gene involved in lignin biosynthesis, *cad*,encoding cinnamyl alcohol dehydrogenase. These GM poplars were made by INRA-AGPF and the field trial has been established in Belgium under the framework of the EU-funded project MultiBioPro. The CAD enzyme catalyses the last step in the biosynthesis of the building blocks of lignin. It was demonstrated previously that CAD-deficient poplar wood, both greenhouse- and field-grown, was easier to delignify in simulated Kraft pulping experiments (Baucher et al. 1996; Lapierre et al. 1999; Pilate et al. 2002). The aim of the current field trial is to investigate the influence of CAD downregulation on the efficiency of wood processing into fermentable sugars and bioethanol when these trees are grown in a short rotation coppice culture, similar to the CCR field trial. The CAD field trial consists of three transgenic poplar lines in which CAD is down-regulated using an RNA interference strategy. The field itself is divided in six randomized blocks per line with 40 clonal replicates in each block. This on-going field trial was planted in May 2014 and a first coppice and harvest was performed in February 2015.

3 Field Tests in Finland (8 Field Trials, 6 with GM Trees)

3.1 Pollination Experiments with Transgenic Pollen (2 Field Trials)

Two approved field experiments in Finland are notable as they were not conventional GM tree field trials but were set up in order to evaluate pollen transformation

as an alternative gene transfer method for Scots pine (*Pinus sylvestris* L.) and Norway spruce (*Picea abies* L. Karst.). The marker genes GUS (β-glucuronidase, *uid*A) and *npt*II (neomycin phosphotransferase) were transferred biolistically to pollen grains (Häggman et al. 1997; Aronen et al. 1998). To enable the seeds to develop under natural conditions, the pollen was transferred to female cones on trees growing in the outdoor seed orchards at the Research Park of the Finnish Forest Research Institute (Metla), Punkaharju. However, all cones and female catkins were covered by specific protection bags for containment of transgenic material (Fig. 2a, b). It was obvious that the bags affected cone development negatively, particularly in the case of Scots pine. The seed production of Scots pine and Norway spruce varied yearly. Based on the GUS assay, 601 of the Scots pine seedlings germinated in the greenhouse were further tested by PCR. One of the seedlings turned out to be transgenic (Aronen et al. 2003; Clapham et al. 2003). None of the Norway spruce seedlings were confirmed to be transgenic.

3.2 Silver Birch (5 Field Trials)

Altogether, five genetically modified silver birch (*Betula pendula* Roth) field trials were established in Finland between the years 1990 and 2013 in three different locations. The first short trial (1990–1991) was established by the company Kemira Oy at Vihti, Kotkaniemi test farm. The purpose of this small-scale trial (60 birches) was to study the effect of the marker genes *npt*II and *lac*Z (beta-galactosidase, EC 3.2.1.23) on birch growth. Even though the results were positive, the field trial was ended in 1991 partly due to the company decision to focus on fertilizer production and also due to corporate acquisition (pers. comm. Prof. Matti Sarvas, Dr. Pauli Seppälä and Dr. Kari Jokinen).

Another early research-oriented GM birch field trial in Eastern Finland at the Research Park of the Finnish Forest Research Institute (Metla), Punkaharju, was originally planned to run from 2000–2005 (Fig. 2d). The purpose of the trial was to study the effect of genetic modification on the key enzymes of carbon and nitrogen metabolism, the Rubisco small subunit gene (*Rbc*S, Kontunen-Soppela et al. 2010) and the nitrate reductase gene (*Nr*), and the marker gene *npt*II (Ryynänen et al. 2002). Unfortunately, the trial was destroyed, the field arrangement was lost and the trial had to be ended.

At the University of Helsinki field experimental site of Viikki, a silver birch field trial was established from 2000–2003 to study biotic interactions between trees, fungi (Pasonen et al. 2005, 2009), and insects, and to evaluate growth characteristics and adaptive traits (Pasonen et al. 2008) and palatability for mountain hare and roe deer (Vihervuori et al. 2012). The policy-relevance of the GM field trial and its public context were also evaluated (Valve et al. 2010). The trial consisted in total of 1050 seedlings from 15 randomly selected chitinase-expressing (Lohtander et al. 2008) and four lignin-modified (Seppänen et al. 2007) transgenic lines along with the corresponding non-transgenic control clone birch lines and eight other

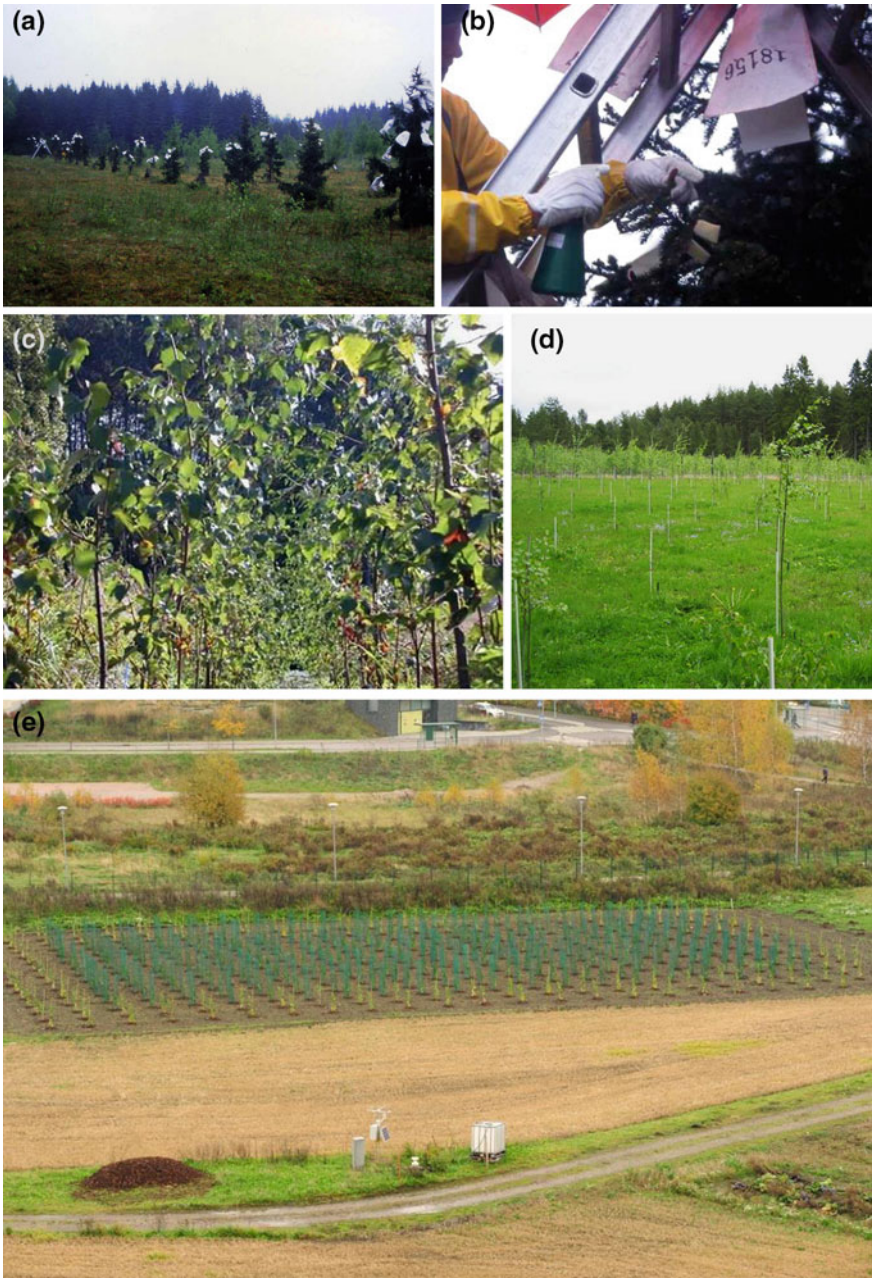

Fig. 2 Finnish GM forest tree field trials. **a** *P. abies* pollinated by GM pollen in 1996 and covered by protective bags (*photo* Teijo Nikkanen), **b** *P. abies* female cone treated with Triton prior to isolation (*photo* Jouko Lehto), **c** Chitinase *B. pendula* at the Viikki field trial (*photo* Ari Pappinen), **d** RbcS *B. pendula* at the Punkaharju field trial in year 2004 (*photo* Jouko Lehto), **e** The effect of enhanced cytokinin and ethylene signalling on wood formation in *P. tremula* × *tremuloides* (*photo* Tuomas Puukko)

non-transgenic "wild" lines (Fig. 2c). Chitinase expressing lines with high or intermediate accumulation of the chitinase transcript were more resistant to birch rust than those showing a low accumulation, but in general GM birch plants were less resistant to leaf spot disease than corresponding control lines (Pasonen et al. 2004). The level of transgene expression did not affect growth or leaf phenology, but instead it influenced parameters related to the stress status of a tree. Some transgenic lines showed deviant performance compared to the control plants but these differences were suggested to be due to position effects of the transgene, not due to the level of transgene expression. Tree genotype was a more important factor determining the structure of fungal communities than the transgenic status of the plants (Pasonen et al. 2009). Thus, the conceivable changes in the litter quality, possibly derived from pleiotropic effects due to gene modification, were either absent or too weak to affect the decomposability of the litter in the soil (Vauramo et al. 2006). One transgenic birch line was significantly less palatable to hares than the wild-type control. The results of this study indicated that plant genotype may be related to the palatability of plant material when transgenic and wild-type woody plant material is used as winter food for hares (Vihervuori et al. 2012). However, the results did not reveal changes in the palatability of the studied transgenic lines that could be related to the functioning of the transgenes.

In Joensuu, at the University of Joensuu (at present University of Eastern Finland) within the area of the botanical garden Botania, a silver birch field trial was established in 2005–2008, and continued until 2012. The purpose of the release was to study environmental effects associated with the prevention of flowering by genetic modification (Lännenpää et al. 2005; Lemmetyinen et al. 2008). Potential unforeseen adverse effects were also a special object of interest in the experiment, and they were studied by examining e.g. changes in growth habit and branching, chemical composition of plants, and plant resistance to herbivores and pathogens. Flowering was prevented by introducing a barnase gene regulated by the promoter (BpMADS5pro) of the inflorescence-specific gene (*BpFULL*1) from silver birch. The marker gene was *npt*II and the transgenic material was produced by *Agrobacterium*-mediated transformation. The experiment included four genetically modified lines of birch clone BPM5. The results indicated that the non-flowering phenotype was stable: none of the individual plants of the transgenic lines had inflorescences during the study period (2005–2012), although the early flowering non-transgenic control line flowered every season. Compared to the wild type, the transgenic lines had slightly slower height growth, increased branching, and smaller leaf size. The increased branchiness was positively correlated with the abundance of spiders on saplings, which may affect the number of herbivorous insects. However, there were no statistically significant differences in visual damage estimates of the lines. The transgenic lines did not differ from the wild type either in chemical composition or in insect feeding assays, but rather, the results varied depending on the transgenic line and insect species.

3.3 Hybrid Aspen (1 Field Trial)

At the University of Helsinki field experimental site of Viikki, a field trial with genetically modified hybrid aspen (*P. tremula* × *P. tremuloides*) clones T89 and 51 was established in 2013 and is scheduled to run until 2018 (Fig. 2e). The general aim of the release is to conduct basic research on hybrid aspen and understand the role and function of the plant hormones ethylene and cytokinin in wood formation. The second major aim is to understand the function of stomatal regulation in hybrid aspen under natural growth conditions. The results are also expected to provide important knowledge for traditional tree breeding.

The transgenes introduced overexpress ethylene response factor (*ERF*) genes or a gene enhancing cytokinin signalling under a constitutive LMX5 promoter, which directs the expression to wood forming tissues. Overexpression of these genes modifies wood properties and enhances growth. In another set of genetically modified hybrid aspens the function of stomata has been modified by silencing the hybrid aspen orthologues of *Arabidopsis thaliana* slow anion channel-associated 1 (*SLAC*1) and *SLAC*1 homolog 3 (*SLAH*3) using transgenes under the control of a guard-cell-specific promoter. Also, the *A. thaliana SLAC*1 gene was expressed in hybrid aspen since poplar *AtSLAC*1 orthologs might have a different function. The hygromycin phosphotransferase (*hpt*) marker gene, conferring hygromycin B-resistance, and/or the neomycin phosphotransferase (*npt*II) marker gene, conferring kanamycin-resistance, were used to select the genetically modified hybrid aspens. Genetic transformation was performed by *Agrobacterium*-mediated transformation.

4 Field Tests in France (7 Field Trials with GM Poplar)

Over the last 25 years, seven different field trials with GM poplar trees have been set up in France, mainly by INRA with one by AFOCEL (now part of FCBA), to explore the behaviour of GM trees in natural conditions.

The first French GM tree field trial was set up in 1991 by the INRA-AGPF (Forest Tree Breeding Research Unit) in their tree nursery near Orléans and lasted for 8 years until 1999 (Fig. 3a). The aim was to assess the stability of transgene expression in a model poplar tree (INRA clone 717-1B4, a *P. tremula* × *P. alba* hybrid) over several years growth and development. Toward this end, expression of a reporter gene driven by a constitutive promoter was measured in many samples (buds, leaves, stems and roots) collected at different seasons and over the years. This first experiment was completed by a second field trial, involving a higher number of transgenic lines in order to evaluate the stability in trees of tissue-specific expression of a transgene over time and space (Pilate et al. 1997; Hawkins et al. 2003). Overall, all these experiments led to the conclusion that transgene expression was stable although highly variable.

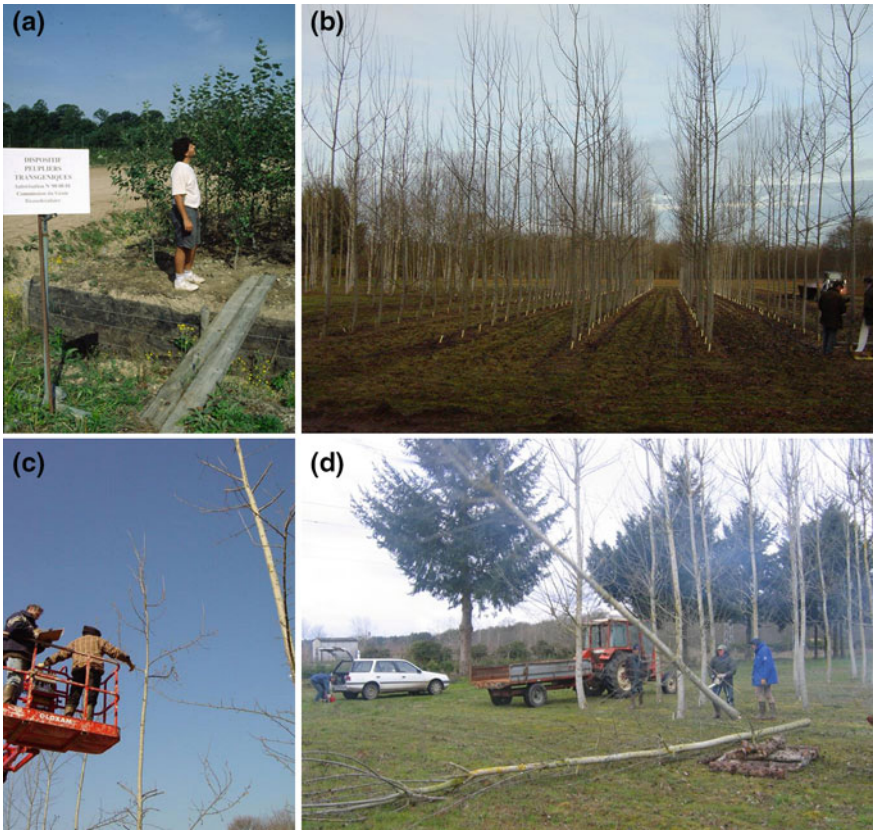

Fig. 3 French field trials as regular poplar plantation. **a** First European small-scale field trial with GM trees set up in 1991. **b** Rows of 5-year-old lignin-modified aspen trees (photo taken February 2004), **c** Catkin removal is easy as flowering takes place before leaf emergence, **d** Final harvest of 10 year-old lignin-modified aspen trees (photo taken February 2007)

The field trial established in April 1994 by AFOCEL evaluated GM poplar cv Hunnegem expressing the *bar* gene which confers resistance to ammonium gluphosinate (a herbicide also known as phosphinotricin or Basta). The transgenic plants were produced in collaboration with M. De Block (PGS, Belgium; De Block 1990). The objective of the trial was to assess the durability of herbicide resistance during a regular short rotation coppice cycle and assess whether initial direct herbicide treatment on the GM trees would insure maximized tree growth in the first year and potentially save on additional treatments in following years (the currently recommended cultural practice). Prior to field installation, the transgenic poplar lines had been evaluated for 2 years in confined greenhouse conditions in order to check for any obvious effects of the genetic modification on tree growth and development. The field trial was set up on an area of 0.8 ha, with 4 blocks of 20 plants per line (3 different transgenic lines and 2 WT lines, Hunnegem and Beaupré)

and per treatment (3 herbicide treatments). The trial was monitored by the Regional Plant Protection Services.

The cv Hunnegem is a male poplar clone (*P. trichocarpa* × *P. deltoides*) that flowers in the field after 8–10 years. The trees were grown as SRC in an agricultural area, with 3 seven-year rotations scheduled, and the first coppicing occurred in 2001. However, the trial was prematurely terminated in 2005. A "safety" zone was regularly maintained around the plantation with a 2 m deep ditch to prevent any risk of sucker spreading beyond the trial site. Each year, herbicide spray tests consistently revealed complete Basta resistance in the transgenic lines, while soil treatment in and around the plantation never revealed any evidence of the emergence of Basta resistant suckers. The observations and measurements were performed at least three times per year during the whole duration of the trial. After 7 years, no flowering was observed and vegetative growth was similar in GM and wild-type trees. The field trial was prematurely terminated in 2005, in part because of the general bad shape of the trees due to severe rust attacks in 2000 and the following years, as Hunnegem trees, whether GM or not, are very sensitive to this disease. For 3 years following the termination of the trail, the plot was carefully monitored and regularly treated with Basta in order to identify potential regrowth of transformed material, but none occurred.

The other five French GM tree field trials were again located at the INRA tree nursery near Orléans and assessed the effects of several different genetic modifications on tree growth and development in near natural conditions in the model INRA clone 717-1B4 (*P. tremula* × *P. alba* hybrid). Unlike the first 1991 trial using marker genes, these later trials involved trees modified for agronomical or technological traits where the objective was to verify that valuable phenotypes observed in the greenhouse were also present under plantation conditions. All field trials were set up and monitored in accordance with the French regulations on GM dissemination and after getting the suitable authorizations (Fig. 4b). Generally, these authorizations were granted provided suitable containment was ensured to prevent any reproductive or vegetative dissemination, irrespective of the transgene present (Fig. 3c).

A trial between 1995 and 1999 evaluated GM poplars transformed with a cysteine proteinase inhibitor gene. The poplars had been shown to be tolerant in laboratory feeding tests to *Chrysomelae tremula*, a coleopteran phytophagous beetle that can cause important damage to poplar nurseries and short rotation coppices (Leplé et al. 1995). In the controlled conditions of a greenhouse, the expression of the cysteine proteinase inhibitor gene translated into an increased mortality of *C. tremulae* fed on transgenic foliage. However, this effect was probably too weak to induce any notable effects on *Coleoptera* feeding on field-grown transgenic trees.

Three field trials were carried out to evaluate GM trees modified for lignin quality or quantity (Figs. 3b, d, and 4a, e). Lignin is a major cell wall component in xylem and fibre cells that provides the mechanical support necessary for large trees to support their own weight, and the impermeability needed for efficient sap conduction between roots and leaves. Variations in lignin content or composition can influence wood technological properties either chemically (for pulp, paper, or



Fig. 4 French field trial as SRC. **a** Plantation of SRC lignin-modified aspens. **b** Field trial inspection by Plant Protection Service (photo taken spring 2008), **c** Sampling of soil cores/roots for assessment of environmental impact of GM modification on soil fungal communities by L. Danielsen and colleagues (photo taken October 2009), **d** Visit of the French GM field trial during a workshop of the COST FP0905 action in September 2011, **e** SRC of lignin modified GM aspens in February 2012 just before harvest

bioethanol production) or physically (mechanically) for solid wood use, and could also potentially lead to major disturbance in tree growth and development, both effects requiring field test evaluation.

These evaluations have been performed on trees down-regulated for any one of 4 key genes from the monolignol biosynthetic pathway, each of them leading to specific modifications in lignin polymer structure and in turn in wood properties. Notably, wood from one transgenic line down-regulated for CAD, the last enzyme from the monolignol pathway, appeared easier to delignify during the pulping process making it possible to use less harsh chemicals to produce, with the same

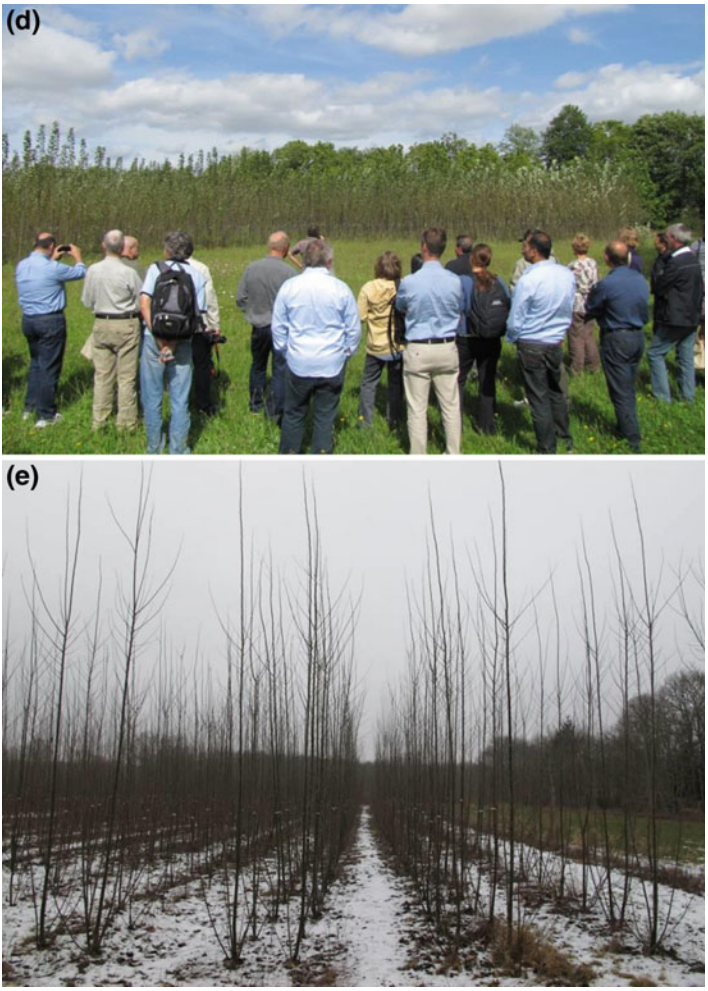

Fig. 4 (continued)

yield, a paper of better quality (Pilate et al. 2002). In addition, the genetic modification did not seem to affect tree growth and development. Other lignin-modified field-grown GM trees were down-regulated in caffeic acid *O*-methyl transferase (COMT), caffeoyl-coenzyme A *O*-methyl transferase (CCoAOMT) or CCR. Several CCR-down-regulated transgenic lines were also superior to wood from control trees for pulping (Leplé et al. 2007) as well as for biofuel production (Van Acker et al. 2014). However, in these CCR-suppressed GM trees, the lignin modifications led to a decreased growth rate most likely due to problems with vessel conductance and sap transport. Other analyses performed on these field trials included global assessment of the effects of genetic modifications on wood

decomposition (Pilate et al. 2002) as well as the effects of lignin modifications on soil and root fungal diversity (Fig. 4c) (Danielsen et al. 2012, 2013).

At the moment, field trials with GM crops and trees have been interrupted in France. This is the case even though 25 years of safe field evaluations of transgenic trees have yielded a lot of new knowledge on the behaviour of GM trees, have evaluated their potential effects on the environment, have identified promising genotypes for more applied studies, and have suggested new routes for producing trees with improved wood for industrial applications such as biofuel production. In addition, during these 25 years of experiments, a lot of expertise was gained in the management of GM tree trials, and how to control vegetative and sexual dissemination. Finally, GM field trials offered a useful platform for communicating directly with the public and the media about experiments with GM trees (e.g. Fig. 4d).

5 Field Tests in Germany (4 Field Trials with GM Poplar)

In Germany, the first release experiment with GM trees was established in 1996 by the Federal Research Centre of Forestry and Forest Products, Institute of Forest Genetics and Forest Tree Breeding, Grosshansdorf. Permission for this field trial was given for five years, ending in autumn 2001. In total, eight transgenic aspen clones comprising two different gene constructs (35S::*rol*C in aspen lines Esch5 [*P. tremula* × *P. tremuloides*], Brauna11, W52 [both *P. tremula*]; and rbcS::*rol*C in aspen line Esch5) and the three control lines were planted out on a field of approximately 1500 m^2 (Fladung et al. 2004; Fig. 5).

The aim of this experiment was to investigate the stability and expression of foreign genes in transgenic trees on a long-term basis under field environmental conditions. The transgenic plants were measured with respect to growth (height, stem diameter, leaf size) and physiological parameters (dormancy, flushing, flower formation, hormone levels, metabolites) (Fladung et al. 2004). Phenotypic instabilities detected in individual plants as reversions to wild type from the dwarfy phenotype of the 35S::*rol*C transgenic trees were also investigated in the lab using molecular methods (Fladung et al. 1996; Fladung 1999; Kumar and Fladung 2000a, 2001; Fladung and Kumar 2002). Based on these results, a quick and easy molecular test was developed to assess the possible stability/instability of a foreign gene in transgenic trees at the genomic level (Kumar and Fladung 2000b).

The mycorrhizal status of the roots was analyzed in the transgenic and non-transgenic trees. No differences were found in the mycorrhizal status between control and transgenic trees with one exception in the transgenic clone Esch5:35S::*rol*C#5 (Kaldorf et al. 2002), however, the ectomycorrhizal spatial distribution was not the same in the different aspen clones (Kaldorf et al. 2004a). The mycorrhizal symbiosis is believed to provide the highest probability for horizontal gene transfer due to its special anatomical features forming the plant/fungus interface. Therefore,

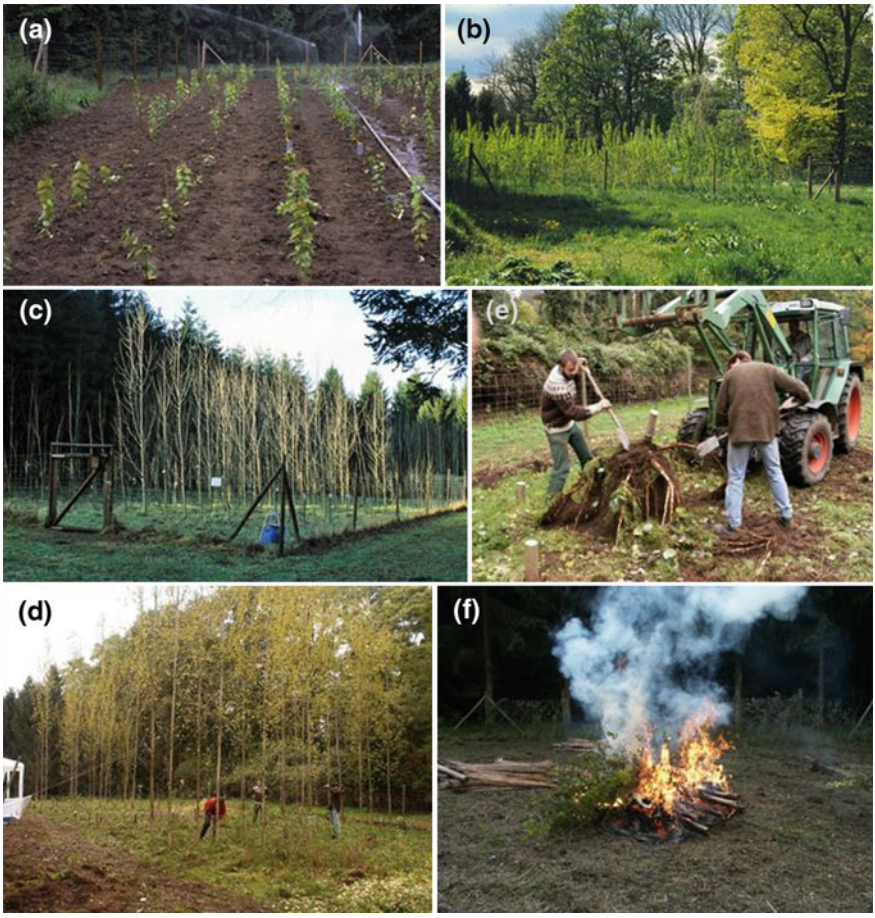

Fig. 5 German field trial as SRC. **a** RolC-transgenic aspen few days after planting. **b** Field trial in spring 1998. **c** Field trial in December 2000. **d** Harvest and sampling of trees in October 2001. **e** Removal of rootstocks from the soil. **f** Burning of transgenic material after clearing the field trial

to unravel possible horizontal gene transfer events, small cuttings of transgenic aspen containing the rbcS::*rol*C gene construct were grown in vitro together with the ectomycorrhizal ascomycete *Phialocephala fortinii*, which was isolated from mycorrhized roots obtained from the field-grown aspen (Fladung et al. 2004). After 12 weeks of co-cultivation, genomic DNA was isolated only from hyphae of the *P. fortinii* mycorrhizas. No *rol*C signal could be detected in any of the analyzed *P. fortinii* colonies (Kaldorf et al. 2004b).

The susceptibility of the leaves to *Melampsora* and *Venturia* was analyzed according to a special evaluation technique. The 35S::*rol*C transgenic trees had a higher degree of susceptibility than the control plants (Fladung et al. 2004). Potential risks of transgene escape via vegetative dispersal were also evaluated. In

the 4th and the 5th year after establishment of the field trial, an increasing number of root suckers could be observed developing from roots of transgenic and non-transgenic trees (Fladung et al. 2003). The phenotype of all suckers was wild type or rbcS::*rol*C. It could clearly be demonstrated that vegetative propagation via root suckering is easily controllable by best practice tree cultivation.

The second field release experiment was mainly designed to quantitate possible horizontal gene transfer from transgenic trees to mycorrhiza fungi under optimized conditions. The field experiment was again initiated by the Federal Research Centre of Forestry and Forest Products, Institute of Forest Genetics and Forest Tree Breeding, Grosshansdorf, in close cooperation with the University of Tuebingen. This experiment was conducted in a closed 1000 m^2 large field station and approved for 3 years from 2000 to 2003. Transgenic aspen trees were generated containing the *Streptococcus hygroscopicus* bar gene conferring herbicide (BASTA) resistance under the control of a fungal GPD promoter (Nehls et al. 2006). Roots containing ectomycorrhiza were sampled twice a year (May and September in 2001 and 2002, respectively). Mycorrhizas were isolated from soil cores under a dissection microscope and morphotyped according to Agerer (1987–1993). In total, 120,000 fungal samples were isolated and transferred to BASTA-containing selection medium.

Out of these, 40,000 isolates were revealed to be BASTA-tolerant and could be divided into two groups. The first group (majority 95 %) contained fast-growing fungi that colonized a Petri dish within a few days and were classified as saprophytic fungi (mainly *Aspergillus*) (Nehls et al. 2006). The second, smaller group (5 %) grew more slowly and morphological and molecular analyses revealed them also to be ascomycetes, representing endophytic but not ectomycorrhizal fungi (Nehls et al. 2006). PCR amplification assays were initiated to search for the presence of the bar gene in all fungal isolates. No bar gene signal could be obtained from any of the slow growing BASTA-tolerant isolates, suggesting that the fungal isolates were naturally herbicide resistant (Nehls et al. 2006).

Two further field trials carried out from the University of Freiburg dealt with the topic of phytoremediation. The experiments took place in Helbra (Saxony-Anhalt, from 2002–2004), and in Helbra and Mansfeld (Saxony-Anhalt 2003–2005) (Peuke and Rennenberg 2006). The transgenic poplars (*P. tremula* × *P. alba*) overexpressed the γ-glutamylcysteine synthetase (*gsh*I or γ-ECS) gene from *E. coli* to enhance glutathione (GSH) levels in the cytosol leading to an elevated capacity for phytochelatins (PC) synthesis. PCs are naturally occurring peptides with good binding affinities for a wide range of heavy metals (Kang et al. 2007). The expression of the *gsh*I gene in transgenic poplars led to a two- to four-fold enhanced GSH concentration in the leaves. In greenhouse experiments, the *gsh*I transgenic poplars revealed a high capacity for heavy metals detoxification of soils.

The capacity of GshI overexpressing poplar for phytoremediation was evaluated in the two field trials at different levels of copper contamination and under different climatic conditions (Peuke and Rennenberg 2006). The transgenic poplars were genetically stable when grown under field conditions. No horizontal gene transfer to rhizosphere microorganisms or to mycorrhiza was detected and no general impact on

the environment could be observed (Peuke and Rennenberg 2006). *gsh*I transgenic poplars possess a higher capacity for accumulation of heavy metals when grown on highly contaminated soils. However, on control sites or sites with low contamination of heavy metals, no differences in phytoremediation capacity could be observed between *gsh*I transgenic and control poplars (Peuke and Rennenberg 2005, 2006).

6 Field Tests in Spain (2 Field Trials with GM Poplar)

The number of field trials with transgenic trees in Spain is very limited. Fifteen records for authorization of field trials are available at the Spanish Commission on Biosafety (http://www.magrama.gob.es), but published data are only available from a few studies including assays of transgenic poplar for increased yield using the hybrid *P. tremula* × *P. alba* 717-1B4 clone, a classical clone in forest tree research used in many studies.

The work of Jing et al. (2004) describes a field trial of transgenic poplar overexpressing a glutamine synthetase gene. This research was carried out by the University of Málaga with the aim of evaluating the stability of transgene expression and to confirm previous results indicating an effect on plant growth rate. Glutamine synthetase is involved in nitrogen assimilation and amino acid biosynthesis, representing a key enzyme in the biosynthesis of nitrogen compounds (Gallardo et al. 2003). Transgenic poplars overexpressing glutamine synthetase were characterized by higher protein, chlorophyll and growth in chamber or greenhouse studies (Gallardo et al. 1999; Fu et al. 2003). The transgenic trees also exhibited increased photosynthetic and photorespiratory capacities, improving their tolerance to drought stress compared with control plants (El-Khatib et al. 2004). The higher production of the glutamine synthetase enzyme also conferred a higher tolerance to phosphinotricin, a common herbicide used in controlling weeds in plantations (Pascual et al. 2008). The results from a 3-year field trial confirmed the previous results obtained in greenhouse studies and the transgenic poplars reached average heights 41 % taller than control plants after the third year of growth (Jing et al. 2004). This study was carried out in the province of Granada (Spain) with plants from eight independent transgenic lines. No flowering was observed during this study, and no apparent effect on nitrate content in soil was observed besides the differences in growth between transgenic and control plants. In addition, no differences in lignin content between transgenic and control plants were observed in 1-year and 5-year-old plants (Jing et al. 2004), but a recent study indicates differences in wood chemistry with respect to control plants in samples from three different lines in a 3-year field study in Virginia (USA; Coleman et al. 2012). Together, the findings suggest a higher capacity of the transgenic trees for both primary N assimilation and re-assimilation of ammonium released in different metabolic procedures, and the potential for improving growth and biomass production of productive clones or other tree species.


(a)
(b)

◄ **Fig. 6** Spanish field trial. Field trial established in Madrid (Spain) in 2012 to test biomass yield under SRC management of genetically modified hybrid poplars (*P. tremula* × *P. alba* INRA clone 717-1B4) developing a high degree of sylleptic branching. The out-door study included the wild type genotype as control, two events over-expressing the RAV1 (Related to ABI3/VP1 1) transcription factor from chestnut, and two events showing a reduced expression of the poplar endogenous genes PtaRAV1 and PtaRAV2. Thirty individuals per genotype were planted into three blocks of 10 plants each. The experimental plot area was 204 m^2, and the chosen plantation density was 10,000 trees/ha. It consisted of 12 × 17 rows with a spacing of 2 × 0.5 m, surrounded by a border row of *P.* × *euramericana* clone I-214 individuals. **a** July 2012 **b** January 2015

The Technical University of Madrid is also carrying out a field trial to evaluate the biomass yield of two events of GM poplars showing a high degree of sylleptic branchiness after over-expression of the chestnut transcription factor *Castanea sativa* RELATED TO ABI3/VP1 (CsRAV1) (Moreno-Cortés et al. 2012). For this purpose, the plantation was established at high density (10,000 trees/ha) and managed under a short rotation coppice system, with irrigation and weed control (Fig. 6). Several growth and biomass productivity determinants were measured before coppicing in 2013, two growing seasons after the establishment of the plantation. One of the *CsRAV*1 over-expressing events showed a ca. 17 % larger basimetric area and dried aerial biomass than non-modified poplars. Histochemical and composition analysis of this modified wood did not reveal any change compared to the non-modified poplars. Calorific value determination also showed that modified and non-modified woods were comparable (Moreno-Cortés et al., unpublished results). This specific genetic modification is potentially applicable to any woody species, taking profit from the adaptive characteristics of each species to a particular habitat.

7 Field Tests in Sweden (8 Field Trials with GM Hybrid Aspen)

The first field trial with GM hybrid aspens in Sweden was established in 1994 in Sävar outside Umeå by researchers at the Swedish University of Agricultural Sciences. This, and subsequent *Populus* field trials in Sweden, have been performed with the hybrid aspen (*P. tremula* × *P. tremuloides*) clone T89, and in comparison with field trials in other European countries the Swedish trials have in general been larger in terms of the number of different genes modified and studied in the same trial. In the first trial, either one of five genes from *A. tumefaciens* affecting auxin or cytokinin metabolism, two genes from *Agrobacterium rhizogenes* affecting root formation, *iaalys* from *Pseudomonas savastenoi*, three genes originating from *E. coli* (*uid*A, *hpt* and *npt*II), one gene from winter flounder coding for an antifreeze protein (AFP) and finally the luciferase from American firefly, were introduced into the plants, The purpose was to characterize how growth in plants was affected by modified hormone metabolism, as well as more general studies on long-term

stability of transgene expression. The AFP protein was included to investigate if chilling tolerance could be modified. The trial led to a fierce discussion in Sweden about the potential risks associated with the GM technology, and in particular the experiment with the winter flounder AFP protein was heavily criticized with the argument that such trees may escape and reproduce uncontrolled in the environment since they potentially could outcompete the native trees. The idea of moving "fish genes" to trees was not appreciated either. However, quite ironically it turned out that genotypes based on the T89 clone, including those containing the antifreeze gene, did not even survive the first winter in the field (in northern Sweden).

The second GM hybrid aspen field experiment, performed in 2004 and 2007 in Umeå, was a short-term experiment with trees in pots transferred to the field only for a short time in the summer, with a gene involved in the regulation of photosynthetic light harvesting (PsbS). The purpose was to see if the consequences of a lack of PsbS, engineered using an antisense transgene, were the same in hybrid aspen as in *Arabidopsis*.

The third experiment with GM hybrid aspen was established at two sites (in Halmstad and Laholm municipalities in southern Sweden) in 2010. In this experiment, 16 genes were selected from large gene finding projects performed by researchers at Umeå Plant Science Centre, in collaboration with the company SweTree Technologies. The basis of the selection was that each of these 16 genes had, upon overexpression or RNAi-mediated down-regulation, resulted in trees that under greenhouse conditions grew faster than the wild type. The aim of the field experiment was to see if the increased growth was transferrable to field conditions, and if this led to any trade-offs in terms of decreased hardiness or increased susceptibility to pests. Three independent lines transformed with each gene construct were included, in total 48 GM lines, and in total (including wild type) ca. 700 trees on each of the sites (Fig. 7). Many of the genes coded for transcription factors, while others had other more or less well defined functions. For all traits other than increased growth, the set could be regarded as a random set of 16 out of over 1000 genes whose expression levels had been modified in the large gene-finding projects. Therefore, this set of 48 clones could also be used to quantify unintentional changes to properties resulting from the transformation event, and for this—as well as demonstration purposes—a set of randomly selected natural genotypes of aspen was planted next to the GM trees on one site. This would allow for a comparison of all variation created unintentionally to the amount of natural variation of the same trait, as the same traits (e g growth, leaf shape, grazing by a wide variety of insects, pathogen infection, frost damage etc.) were measured on all trees. The establishment of the field experiment led to some media debate, NGOs were invited to participate in a discussion about the setup (but did not show up), background information was published (http://www.upsc.se/about-upsc/other-information/gmo-information.html), an information meeting was held and despite a few statements in local media, the experiments have been performed uninterrupted. The experiment has recently got a permit for a 5-year extension. The permit stipulates that a fence should surround the experiment (10 m from the trees), that native aspens 50 m from the fence should be removed as well as suckers between the trees and the fence.



Fig. 7 Swedish field trial. The GM hybrid aspen field trial established in Laholm, Sweden 2010. Ca. 800 trees (16 transgenes × 3 independent transformation events for each gene = 48 lines, plus wild type T89) with modified growth properties. *Photo* Stefan Jansson

The site should be regularly inspected for flowering and—if flowering is detected (which has not happened so far) all ramets of the flowering genotype should be removed.

Based on the experiences from this experiment, several others have been initiated at the same site (in Laholm). One rather similar experiment was initiated in 2011, led by researchers at the Swedish University of Agricultural Sciences, but with seven genes where modification of expression levels had resulted in altered wood properties under greenhouse conditions. Experiments led by SweTree Technologies, with 14 genes where altered expression levels had led to increased growth in the greenhouse, or which may influence drought tolerance, were initiated in 2011, and an experiment with 7 genes in 2012. Similarly, two experiments were started in 2014, one with trees with modified expression of 21 genes involved in the regulation of wood properties, and one with 27 genes potentially regulating phenology. At the Laholm site there are at present six experiments ongoing, in total covering almost 5 ha and with ca. 3000 trees with modified expression levels of over 90 different genes, the vast majority from *Populus* but a few originating from other species.

8 Field Tests in United Kingdom (2 Field Trials with GM Poplar)

Although there have been five separate notifications of GM tree trials in the United Kingdom (http://gmoinfo.jrc.ec.europa.eu/overview/GB.asp), only one has been reported on fully in the scientific literature (Pilate et al. 2002; Halpin et al. 2007). All of these trials were approved between 1993 and 1996 and involved eucalyptus, poplar or apple. Two approvals concerned the trialling of marker gene systems in trees, one involved the introduction of glyphosate tolerance, and two focused on the alteration of lignin biosynthesis. The lignin modification trials were planted one year apart at the same location at Syngenta's Jealott's Hill International Research Station in Berkshire (Fig. 8) in collaboration with similar trials by INRA in Orléans, France, within a research project with part-funding from the European Commission (FAIR CT95-0424). The trees grew for 3 and 4 years but both trials were ended abruptly in 1999 when the trees were vandalized and chain-sawed by activists. This likely explains why no further trials of trees have been performed in the UK, and research that the country was world-leading in has largely moved elsewhere.



Fig. 8 United Kingdom field trials. The lignin-modified GM hybrid aspen field trials were established at Jealott's Hill, Berkshire in 1995 and 1996 and grew for 4 or 3 years respectively until destroyed in 1999. Front trial block: 72 trees planted in 1995 in a randomized block design included two CAD antisense events and two COMT antisense events (12 ramets per event) plus 24 wild type 717-1B4 trees. Rear trial block: 80 trees planted in 1996 in a randomized block design included a CAD antisense event and a COMT antisense event (24 ramets per event) plus 40 wild type 717-1B4 trees. (*Photo* Claire Halpin)

Results of the longer four-year trial have been presented in several papers (Pilate et al. 2002; Halpin et al. 2007) and are discussed below.

Ironically, it was only evaluation of some of the ecological and environmental impacts of the trial, such as investigation of mycorrhizal associations that was prevented by the attack on the trees. The more applied objectives of the trial were unaffected and evaluation of wood from the trees for stability of gene suppression and lignin manipulation, and the utility in paper production, proceeded as planned (Pilate et al. 2002). Two types of transgenic plants suppressed in two different lignin biosynthesis genes and originally produced by INRA, were present in the trial. Some were suppressed in expression of caffeic acid/5-hydroxyferulic acid *O*-methyl transferase (COMT), while others were suppressed in expression of CAD (CAD). The trial was a sister trial to one performed at the same time by INRA near Orléans, France, on the same clonal trees. As has already been described earlier in this chapter for the trees in the French trial, the UK-grown trees grew normally but had modifications to lignin structure. The trees with reduced CAD expression were easier to delignify so that either less chemical could be used or more pulp could be recovered (Pilate et al. 2002). Gene suppression appeared to be stable over the 4 years of growth and at final harvest CAD enzyme activity was still reduced by 53–85 % of wild-type levels in the CAD-suppressed lines, while COMT activity was reduced by 56–68 % in the COMT-suppressed lines.

The UK trial was constantly monitored to determine whether the lignin-modified trees had altered biological or ecological impacts. Transgene escape was not possible since the trees were *P. tremula* × *P. alba* female clones (i.e. without pollen) and were harvested before any could flower. The incidence of visiting and resident insects was regularly monitored and visible damage and disease symptoms were recorded. All trees attracted a wide variety of insects but feeding damage to the trees was very modest and there was no significant incidence of disease other than occasional low frequency rust on individual leaves (Halpin et al. 2007). Neither insects nor disease affected the transgenic trees any more than the wild-type trees, and all trees remained very healthy throughout the four-year trial (Pilate et al. 2002).

The influence of modified lignin on soil microbiota and properties, and on CO_2 evolution and decomposition, were also considered using samples collected after the termination of the trial. When chopped roots were buried in a test soil over 5-months, slightly more CO_2 was emitted from the transgenic root samples compared to the wild type, indicative of a small increase in the rate of decomposition. The effect was greatest in the first month when labile polysaccharides, not lignin, would be decaying, suggesting that the modified lignin was less effective at protecting other cell wall components from microbial attack than wild type lignin is (Pilate et al. 2002). A longer study set up with trunk wood from the field trial harvest showed no statistically significant differences in CO_2 evolution or mass loss between the transgenic and wild-type samples (Tilston et al. 2004). Variation between samples of the same genotype was greater than variation between genotypes. There were no significant differences in C, N or microbial biomass between soil samples taken from beneath transgenic or wild type trees (Pilate et al. 2002).

Although microbial basal respiration was lower in soils from under two transgenic lines, this did not correlate consistently with transgene expression, and no significant differences in the substrates supporting microbial growth were evident between any of the soils. However, soil taken from a grassy area less than 4 m from the trees was significantly distinctive from the soil under the trees in all properties measured. The cumulative data from this trial suggests that plant residue decomposition and soil properties are more influenced by the type of plant grown and by field environmental variability than they are by genetic modifications to lignin (Hopkins et al. 2007).

9 Conclusion

Of nearly 800 GM tree field trials that have been approved to date worldwide, it is notable that only a very small number, perhaps fewer than 50, have been approved in Europe (Häggman et al. 2013). Indeed, most experiments involving GM trees—as for GM plants in general—do not go beyond the greenhouse stage as it is assumed that this is enough to assess the function of a gene in a biological process, at least for basic research purposes. Setting up any field trial requires time, money, space and technical skills, as well as the tenacity to navigate the considerable hurdle of assembling an application strong enough to gain regulatory approval. For GM trees, there are extra burdens as the evaluation of perennials needs to last for several years, and there are additional constraints related to the regulations on GM confinement to prevent dissemination of the transgene. Despite 25 years of safe field trials, these burdens only seem to be getting heavier (Walter et al. 2010). Taking all of these issues into account, it is easy to understand why most studies of transgenic trees are only performed in greenhouse conditions and European GM tree field trials remain the exception.

The GM tree field trials that have been set up in Europe have addressed numerous important scientific objectives, including evaluation of tree-specific traits (lignin and wood properties, flowering/sterility, transgene expression stability over several years), assessment of general agronomic traits (biomass, resistance to biotic and abiotic stress) and consideration of biosafety issues (horizontal gene transfer, possibility of vegetative dispersal, effect on biotic interactions) (see Table 1). Most experiments have been carried out on poplar, the model species for tree biology, and especially one clone that is easy to transform, INRA 717-1B4 (*P. tremula* × *P. alba*) and also to some extent on the clone T89 (*P. tremula* × *P. tremuloides*). These field trials can all be considered as confined trials as they were set up with strict controls on dissemination and usually terminated before sexual maturity. They were valuable tools for basic research, and for increasing our understanding of some tree-specific biological traits (dormancy, phase change, wood formation, etc.). Besides, they were also the first step towards more applied research and were, for example, essential to produce wood in sufficient amounts for industrial evaluation, under environmental conditions close to those of regular tree field trials. Field tests

are an obligatory early step towards future commercial deployment, although, in Europe, no commercial deployment is foreseen in the short or mid-term. It is noteworthy that even though all experiments performed so far involved relatively small-scale field studies, a long way from any commercialization, none of the results detected any unforeseen risks or effects. Yet several trials were illegally destroyed by anti-GMO activists, and the burden to obtain authorization becomes heavier and heavier, despite the fact that virtually all of the concerns raised against GM trees are related to the commercial deployment stage, and contained field trials are the way to explore the evidence base for those concerns.

In this context, and to return to the question posed in the introduction, what did we really learn from these experiments?

A major outcome was gaining the knowledge that the expression of both silencing and gene-expression transgenes is generally stable over time even in long-lived perennial species such as trees, albeit sometimes variable in level. This has been most easily studied using marker genes such as bacterial *uid*A and *npt*II driven by a constitutive or tissue-specific promoter (Hawkins et al. 2003), or by using *rol*C, whose expression is associated with a very strong phenotype that is easy to monitor (Kumar and Fladung 2001). These studies led to the conclusions that transgene insertions were very stable as was transgene expression. Similarly, barnase expression from an inflorescence-specific promoter prevented flowering in silver birch over several years (Lännenpää et al. 2005), and poplars expressing glutamine synthetase grew taller over 3 years (Jing et al. 2004). Silencing transgenes appear to be equally stable, with the phenotypic effects of silencing a specific gene (e.g. modified lignin) persisting over several years of a trial and target enzyme activity remaining reduced (Pilate et al. 2002).

The trials also increased confidence that the phenotypic effects of transgenes initially evaluated in controlled greenhouse conditions, are frequently sufficiently robust to persist in fluctuating natural field environments, as evidenced by many of the examples described in this chapter. This validated several genes as real commercial targets for improving wood for industrial applications and enabled initial evaluation of the benefits likely to accrue. So, for example, down-regulation of CAD and consequent specific changes to lignin structure led to an increase in poplar pulp yield, or to an opportunity to make cost savings by using less pulping chemicals, with associated environmental benefits. Importantly, in this and many other field trials where growth modification was not the object of the modification, the GM trees showed no significant differences in growth compared to control trees. On the other hand, several deliberate strategies successfully led to valuable biomass increases in poplar. Although robustness of phenotype between greenhouse and field was generally the case, it was not always so. For example, transgenic poplars expressing a proteinase inhibitor were shown to tolerate insect attacks in greenhouse experiments (Leplé et al. 1995) but the effect was rather weak and was no longer significant in natural field conditions in France (unpublished results). In an example from the USA (see chapter by Strauss et al.), poplars down-regulated for 4-coumarate-CoA ligase (4CL, a key lignin biosynthesis gene) exhibited a sizeable growth increase in the greenhouse (Hu et al. 1999), but when similar plants were

independently produced and grown in a field trial, they showed significant reduction in growth (Voelker et al. 2010). Crucially, the results obtained in the field can stimulate important new research directions. For example, poplars down-regulated for CCR produced wood that was more easily processed into ethanol, but the trees had reduced biomass yield, most likely because of xylem collapse and water conduction defects (Van Acker et al. 2014). Down-regulation of CCR would remain a valuable strategy if the yield penalty could be overcome, and this may be possible by targeting gene down-regulation to fibres only, using suitable promoters.

Although virtually all GM field trials enable evaluation of the robustness of genetic modifications over time, very few experiments have evaluated the robustness of the modifications and general field performance across different sites. Three duplicated field trials where the same lignin modified poplars were installed in France and in England (Pilate et al. 2002), in Belgium and in France (Van Acker et al. 2014) or in two sites in Sweden, allow such comparison. All revealed results to be predominantly and qualitatively similar at different sites, just varying occasionally in degree. Multisite field trials certainly deserve to be more frequently pursued, in just the same way that they are recommended for testing new tree varieties created through classical breeding.

In terms of new knowledge gained on the ecological, environmental and biosafety issues of growing GM trees, the European field trials have provided some of the best data to date from which conclusions can be drawn that are actually substantiated by evidence. Where microbial or fungal communities beneath GM trees were evaluated, they showed no significant differences to those under wild type trees, and the type of vegetation (Pilate et al. 2002) or the background genotype (Vauramo et al. 2006) was more important in determining the structure of soil communities than the transgenic status. In general, the degree of variation between GM trees and wild type trees is smaller than variations between different wild type genotypes when grown in the fluctuating conditions of the natural environment (Danielsen et al. 2012; Jansson et al. ms in preparation). Chitinase-expressing silver birch litter was not altered in decomposability (Vauramo et al. 2006) while even roots with modified lignin displayed only a small increase in the rate of decomposition (Pilate et al. 2002). No horizontal gene transfer could be detected between *rol*C expressing poplars and mycorrhiza (Kaldorf et al. 2004b; Nehls et al. 2006) and vegetative dispersal via root suckers either did not occur or was easily controlled by conventional means (Fladung et al. 2003). There was no evidence for significant unanticipated effects in any of the trials. We should emphasize that there has been no study in Europe on gene flow (vertical gene transfer), as in most cases, authorization for field trial was only given provided there was no dissemination of GM material into the environment from pollen, seeds, or suckers. Wind or insect pollinated species certainly have different requirements with respect to isolation distances. However, this will not be so great an issue for short rotation coppices where trees are harvested before being able to flower. The significance of these risks (of dissemination) remains an open question that deserves to be addressed in some cases when the genetic modification may confer an increased fitness to the GM tree (e.g. pest or pathogen increased tolerance). Paradoxically, conventionally bred

commercial poplar clones are not assessed for risk in the same way, despite many being bred to have high resistance to rust, which is a clear fitness advantage. Given that many GM traits only confer an advantage under managed growth conditions or in subsequent industrial processing, only a few traits can be conceived of that might result in increased fitness compared to natural ecotypes. Given that (1) the anticipated variation generated in GM trees is often within the natural variation of the species, (2) any unanticipated effects are even smaller, and that (3) natural variation within a species is in no respect regarded as a risk, nor are traits introduced by conventional breeding irrespective of their influence on fitness, we may seriously wonder whether the huge effort and expense put into some risk evaluations of GM trees is fully rational or justified.

Looking to the future, new technologies are currently under development in plants, including trees (see chapter by Alburquerque et al.), involving nucleases associated with site-specific recognition systems: these technologies allow for specific targeted genes to be mutated, even knocked-out, avoiding the need for incorporation of silencing transgenes, and may enable targeted transgene insertion. These systems can avoid many of the issues that led to criticisms of conventional T-DNA transfer such as the randomness of insertion sites, and the need for integration of additional alien DNA such as selectable marker genes etc. Although, these new technologies (Meganucleases, ZFN, Talen, CrispCAS9) seem very promising, they also need to be carefully assessed, notably for the fidelity of their recognition mechanism. We believe that all new plant material modified for a given trait deserves to be evaluated in a similar way irrespective of whether it was produced by new plant breeding technologies, by classical gene transfer techniques or by traditional breeding (Strauss et al. 2009; Walter et al. 2010): indeed, what matters is the trait modification achieved and not the means of obtaining it.

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Lessons from Two Decades of Field Trials with Genetically Modified Trees in the USA: Biology and Regulatory Compliance

Steven H. Strauss, Cathleen Ma, Kori Ault and Amy L. Klocko

Abstract We summarize the many field trials that we have conducted in the USA beginning in 1995 and continuing to this day. Under USDA APHIS federal regulatory notifications and permits, we have planted nearly 20,000 trees derived from approximately 100 different constructs in more than two dozen field experiments. The large majority of the trials were in *Populus* and included hybrid white poplars (*P. tremula* × *alba* INRA 717-1B4 and *P. tremula* × *tremuloides* INRA *353-53*), but also included diverse hybrid cottonwoods such as *P. trichocarpa* × *deltoides* and *P. deltoides* × *nigra*. One field trial used transgenic sweetgum (*Liquidambar*). Most trials were conducted on Oregon State University (OSU) land, but several were also conducted on the land of industry collaborators in Oregon, Washington, and other states. The main traits we have studied are floral sterility and flowering time modification; size and growth rate modification by gibberellin perturbation; activation-based gene tagging; stability of reporter gene expression and RNAi suppression; herbicide and pest resistance gene impacts on plantation productivity; lignin modification and its impacts on physiological processes; and effects of isoprene reduction on growth and stress tolerance. The most significant lessons from these years of trials are: (1) Visual abnormalities in form or growth rate due to the transformation and in vitro regeneration (somaclonal variants) have been observed in several experiments, but are extremely rare (below 1 % of events produced). (2) Gene expression and RNAi-induced gene suppression have been highly stable —with a virtual absence of transgene silencing—over many years for virtually all transgenic trees whether assayed by a visual phenotype (reporter gene, flowering time, sexual sterility, herbicide or pest tolerance), or by molecular measures of transgene expression (e.g., quantitative RT-PCR). (3) The regulatory process has largely been efficient and workable, though it imposes significant biological constraints, costs, and risks that are very difficult for an academic laboratory to bear when trials span several years. It is most difficult where flowering is

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needed. (4) Field environments invoke complex and largely unpredictable changes to expression and associated phenotypes when studying physiology-modifying transgenes, including those affecting wood properties, suggesting the need to study several field sites, genetic backgrounds, and gene insertion events over many years, similar to common practices of conventional breeding. However, regulatory requirements make this very difficult to do for transgenic trees. (5) Collaborative field trials with industry have shown that common transgenic traits, such as herbicide and insect resistance, can have large productivity benefits in near-operational plantation conditions (e.g., two-year volume growth improvements of $\sim$20 %)—suggesting that it could be highly beneficial to incorporate transgenic traits into production programs. Regulatory reforms to focus on product benefits as well as risks, and that do not assume harm from the use of recombinant DNA methods, are needed if transgenic technology is to provide significant benefits in forestry.

1 Introduction

During the span of our research program, society has gone from a position of great enthusiasm for use of transgenic plants in agriculture and forestry, to one where regulatory, market, and social barriers have grown to the point that transgenic studies are increasingly difficult to fund and carry out (e.g., Viswanath et al. 2012). Very few academic research programs conduct field studies with transgenic trees anymore as a result of these barriers. We have planted more hectares of transgenic trees than any public sector research program in the USA (Biotechnology 2014). However, our program has nearly been shut down several times due to a lack of adequate funding to support the substantial costs of regulatory compliance. Whether the pendulum will swing back or not is unclear, especially as regulations and international market barriers are very slow to change. But, our experience and insights from field studies with transgenic trees, including problems and opportunities missed, may be of value to inform society about whether and how to ease restrictions in the future. Providing these lessons in a single, easily accessible place is the main reason that we have written this chapter.

Our previous summaries of field experience, and the reasons that we believe extensive field research is essential for progress in tree molecular biology and biotechnology, can be found in several review and analysis papers published earlier (Bradshaw and Strauss 2000; Brunner et al. 2004; Strauss et al. 2004; Busov et al. 2005a, b; Valenzuela and Strauss 2005; Wei et al. 2006; Strauss et al. 2009a, b; Strauss et al. 2010; Voelker et al. 2010; Elorriaga et al. 2014). The different types of studies we have carried out over the last two decades are summarized in Table 1, and illustrated in Figs. 1, 2, 3 and 4.

Table 1 List of field trials

Trial name and clones used	Promoter/transgene/terminator	Years	No. trees	Publications
Flowering modification				
First generation sterility				
717, 353	TA29::Barnase::NOS	1995–2009	228	Elorriaga et al. (2014)
	35S::LEAFY::NOS			Skinner et al. (2000), Rottmann et al. (2000)
	APETALA1::GUS::NOS			
	APETALA1::DTA::G7			
	TTS-1::Barnase::35S			
	TTS-1::GUS::35S			
	DTA::NOS::35S			
	SLG::DTA::NOS			
	TTS-1::DTA::35S			
Second Generation sterility—PTD				Elorriaga et al. (2014)
717, 353	PTD::GUS::NOS	2000–2009	229	
	AP3::DTA::NOS			
	35S::GUS::ST-LS1::RUBP			
	ACTIN2::GUS-ST-LS1::RUBP			
	ACTIN11::GUS-ST-LS1::RUBP			
	35S::PTLF::NOS			
Second generation sterility—DNM				
353	ACT11::PTD-1::E9	2001–2009	280	–
	ACT11::PTD-2::E9			
	ACT11::PTAG2-1::E9			
	ACT11::PTAP1.1b-1::E9			
	ACT11::PTAP1.1b-2::E9			
Sterility trial: overexpression/suppression				
717	35S::PMFT-IR::OCS (delay)	2003–2009	202	Mohamed et al. (2010)
	35S::PMFT::35S			
	35S::PCENL1::35S			
	35S::PCENL1-IR::OCS (promote)			
Attenuation sterility trial				
717	PTLF::GUS::G7, MARs	2003–2009	588	Wei et al. (2007)
	35SBP::BARSTAR::E9, MARs			
	35SBPW::BARSTAR::E9, MARs			
	NOS::BARSTAR::E9, MARs			

(continued)

Table 1 (continued)

Trial name and clones used	Promoter/transgene/terminator	Years	No. trees	Publications
	PTLF::BARNASE::G7 35SBP::BARSTAR::E9, MARs			
	PTLF::BARNASE::G7 35SBPW::BARSTAR::E9, MARs			
	PTLF::BARNASE::G7 NOS::BARSTAR::E9, MARs			
	35S::GUS::E9, MARs			
	35SOmega::GUS::E9, MARs			
	NOS::GUS::E9, MARs			
Third generation sterility				
717, 353, 6K10	35S::AG::E9, MARs	2011—current	3,539	Klocko et al. (2014b)
	35S::AP1-M2::E9, MARs			
	35S::AP1-M3::E9, MARs			
	35S::PAGL24-IR::OCS			
	35S::PTFT1-IR::OCS			
	35S::PFT1/PAGL20-IR::OCS			
	35S::PtFT-PAGL20-IR::OCS/35S::PfPFL1-IR::OCS			
	35S::PTAG-IR::OCS			
	35S::PTAG-IR::OCS, MARs			
	35S::PTAP1-IR::OCS			
	35S::PTAP1-PTAG-IR::OCS			
	35S::PTAP1-PTLF-IR::OCS			
	35S::PTD-IR::OCS			
	35S::PTLF-PTAP1-PTAG-IR::OCS			
	35S::PTLF-IR::OCS			
	35S::PTLF-PTAG-IR::OCS			
	35S::PAGL20-IR::OCS			
	35S::PAGL24-IR::OCS			
	35S::PFPFL1-IR::OCS			
	35S::PFPFL2-IR::OCS			
	35S::PSVP::OCS			
	35S::PTLF-IR::OCS/35S::PTAG-IR::OCS			
Sweetgum sterility trial				
Liquidambar styraciflua CV *Worplesdon*	35S::LAG/LSAG-IR::NOS	2007—current	328	–
	En35S::AG-M3::E9, MARs			
	PTD::BARNASE/35S::BARSTAR, MARs			

(continued)

Table 1 (continued)

Trial name and clones used	Promoter/transgene/terminator	Years	No. trees	Publications
Management				
Herbicide resistance stability trial				
717, 353	pTA29::BARNASE::NOS, pSSUARA-TP::BAR::G7	1997–2006	384	Li et al. (2008)
Glyphosate-resistance screening trail				
50-197, 189-434, 195-529, 311-93	FMV::CP4::T9, FMV::GOX::NOS	1996–1999	1,176	Ault et al. (2016) in press
Glyphosate-resistance management trail				
95-529, 311-94	FMV::CP4::T9, FMV::GOX::NOS	2000–2003	1944	Ault et al. (2016) in press
BT screening trial				
24-305, 50-197, OP-367, 189-434	35S::cry3Aa::orf25	1998–2002	502	Klocko et al. (2014a), Meilan et al. (2000a, b)
BT large-scale trial				
OP-367	35S::cry3Aa::orf25	1999–2002	402	Klocko et al. (2014a), Meilan et al. (2000b)
Form and growth rate				
Semi-dwarfism trial				
717	35S::ptaGA2ox::OCS 35S::2-Oxidase::NOS	2003–2008	882	Etherington et al. (2007), Zawaski et al. (2011)
	GAI::GAI (from Arab cDNA, wt-gene)::GAI GAI::gai (from Arab cDNA,51-bp in-frame deletion)::GAI			
	35S::GAI (from Arab genomic, wt-gene)::35S			
	35S::gai (from Arab genomic, 51-bp deletion)::35S			
	35S::rgl1 (51-bp, in-frame deletion)::NOS			
GA competition and yield				
717	35S::GA2-oxidase::NOS	2006–2008	2400	Elias et al. (2012)
	35S::Atrgl-1::NOS			
	35S::AtGAI::35S			
	35S::PtaGA2-ox::OCS			
	NativeGAI:: AtGAI::nativeGAI			
	NativeGAI::Atgai::nativeGAI			
GA growth enhancement				
717	GA20ox7::GA20ox7:: GA20ox7	2008–2010	429	Lu et al. (2015)
GA signaling modification				
	Empty vector			
	35S::AtSPY::OCS			

(continued)

Table 1 (continued)

Trial name and clones used	Promoter/transgene/terminator	Years	No. trees	Publications
	35S::HvSPY::OCS			
GA modification growth trial				
717	35S::GA2oxidase2,7::OCS	2009–2011	502	Lu et al. (2015)
	35S::GA2oxidase1,6::OCS			
	35S::GA2oxidase3::OCS			
	SHI1::SHI1::SHI1			
	PHOR1::PHOR1::PHOR1			
	GA2ox1::GA2oxidase2::NOS			
	RGL1-1::GA20oxidase2::NOS			
	Empty vector			
Phytochrome trial				
717	35S::PHYB1::OCS	2004–2013	220	–
Activation tagging				
Activation tagging trial				
717	35S::none::none	2003–2009	2564	Busov et al. (2003)
Activation tagging trial				
717	35S::none::none	2007–2009	1783	Busov et al. (2010)
Tools and stability				
Alcohol inducible				
717	alcA::GUS	2005–2009	40	–
	35S::PHYB2::OCS			
Transgene stability trials				
717, 353	35S::BAR-IR::OC	2004–2007	340	Li et al. (2008)
	35S::BAR-IR::OC, MARs			
	35S::rbcSp-IR::OC			
	35S::rbcSp-IR::OC, MARs 35S::GFP::35S rbcs::TP::BAR::G7 35S::GFP::35S rbcs::TP::BAR::G7, MARs	2003–2006	3416	Li et al. (2008, 2009)
Other physiology modifications				
Lignin modification				
717	Pt4CL1P::4CL1::NOS	2005–2009	97	Voelker et al. (2010, 2011a, b)
Isoprene reduction trial				
717	35S::PcISPS-RNAi::OCS	2012—current	504	–
All trials	**Total trees**	22,979		



Fig. 1 Sexual sterility field trials. **a** 9-acre sterility trial (photo taken in spring 2013), **b** Dr. Steve Strauss with the same sterility trial showing a block with transgenic clone INRA 353-53 (*P. tremula* × *P. alba*; photo taken spring 2014), **c** Program Manager Liz Etherington inside a coppiced clone bank used to produce cuttings for the same sterility trial, **d** collecting catkins in an earlier sterility trial in ∼1995, and **e** seven-year-old sterility trial of trangenic sweetgum trees showing their fall color

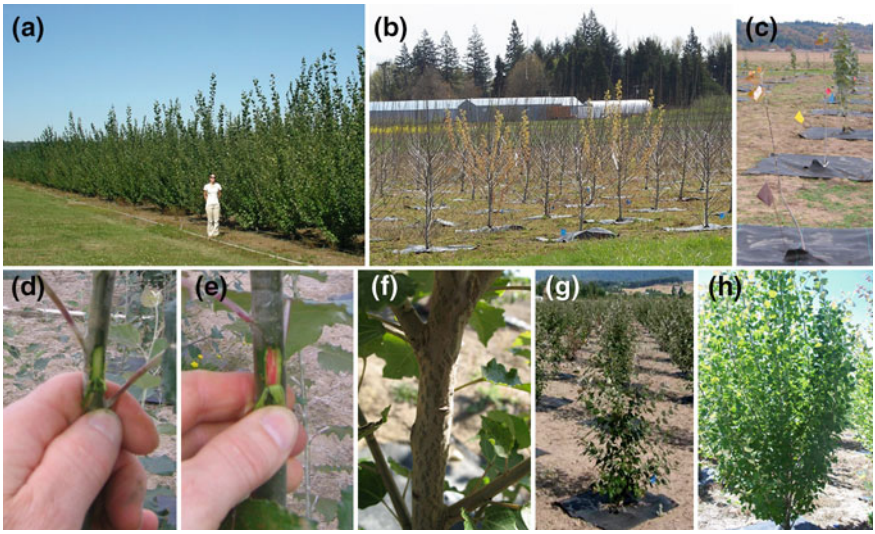

Fig. 2 Gene tagging. **a** Program Manager Liz Etherington with field trial of activation-tagged trees in 2009. A variety of phenotypic alterations were observed in these field trials, including, **b** early bud flush, **c** early leaf senescence, **d**–**f** altered wood color and bark texture (**d** is wild type), and **g**–**h** changes in tree form (more upright branches in **h** compared to wild type in **g**)


(a)
(b)
(c)
(d)
(e)
(f)
(g)
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◀ **Fig. 3** Management trials. **a**–**e** Herbicide resistance field trails of poplars showing **a** rows which have been sprayed with glyphosate. A variety of insertion events with high resistance are shown on the *left*, and the row of dead non-transgenic control trees are on the *right* indicated by the *red arrow*. **b** Glyphosate application directly onto trees. **c** Glyphosate-resistant trees growing well after multiple direct sprays and nearly 2 years in the field. **d** Conventionally grown poplar showing weed proliferation. **e** Glyphosate-resistant poplar with much less weed competition from same experiment as in **d**. **f**–**g** Insect-resistant poplars showing **f** Bt poplar with no insect damage from cottonwood leaf beetle. **g** Non-transgenic control poplar tree from the same experiment as (**f**) showing extensive insect damage, and **h** comparable growth and morphology between Bt trees (*right*) and unmodified trees (*left*) after two growing seasons

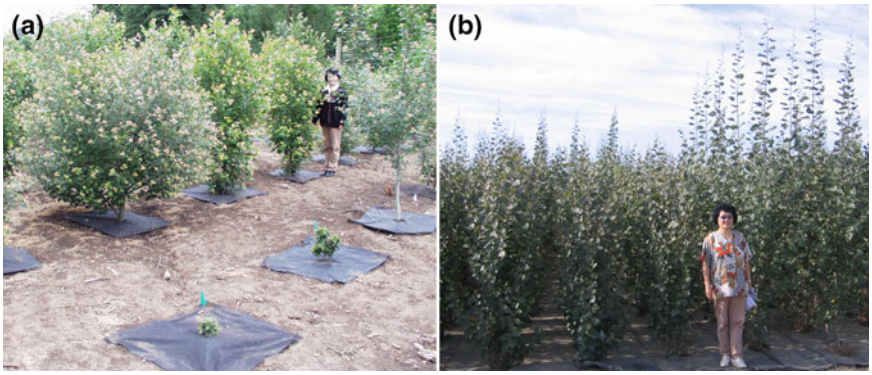

Fig. 4 Form and architecture modification. Dramatic alterations in **a** tree shape (bushy vs. narrow, *back row*) and size (dwarf, *front row*) and **b** stature of GA-modified poplars

Our laboratory has focused on poplars, mostly using model genotypes obtained from collaborators at INRA in France (G. Pilate and L. Jouanin provided hybrids 717-1B4 and 353-53) that are easy to transform and perform well in the field in the Pacific Northwest of the USA. We have also been successful in transforming many other poplar genotypes, but with greater difficulty. We have been able to use transformation as a routine tool, enabling us to produce and test tens of thousands of transgenic gene insertions, called "events." However, for many other tree species and most genotypes, including the closely related willows (*Salix*), transformation remains a costly or extremely difficult tool to use, requiring optimization and trial-and-error protocol development in each laboratory and for each genotype. Unfortunately, as a consequence of the stigma over transgenic methods there has been very little public investment in development of science-based, generalized transformation methods. In contrast, great and often proprietary advances have been made in the private sector. With the extraordinary advances in developmental biology, it is likely that transformation barriers can be much reduced to support a next generation of transgenic tree biology and field testing, if society chooses to reinvest in the area.

In this review we will first summarize the mechanics of high throughput transformation and field trials, including the management challenges and surprises we have run into. Many of these are common to any field trials, but take on additional importance given the high cost and regulatory oversight of transgenic materials.

2 The Lab to Field Pipeline

Because of the ease and reliability in response of the in vitro propagated poplar genotypes that we have used, it has been easy to standardize media and steps in transformation, subculture, and propagation. As a result, most of the steps can be performed by high school or undergraduate students after a modest period of training. The methods we employ are variations of the well known "leaf disc" type of organogenic transformation (Horsch et al. 1985) where sterile in vitro leaf disks, internode sections, and sometimes petioles are cocultivated with *Agrobacterium*, then sequentially placed in callus induction medium, shoot induction medium, and rooting medium in the presence of a selective antibiotic or herbicide. Further propagation to produce the number of clonal replicate trees (ramets) needed for field trials are developed by further shoot development and rooting of nodal segments. Once roots are produced, plants are transplanted to soil and exposed to ambient conditions in a greenhouse slowly over a period of one to two months. After a further one to two months of growth and acclimation in the greenhouse, they are either planted directly in the field, grown out of doors for some weeks to enable further hardening, or induced to go dormant in a greenhouse or out of doors then planted as dormant materials (whole plants or stem sections). While poplar trees can be established using dormant plants or cuttings, most of our trials have been planted with continuously growing materials; the degree of hardening prior to planting depends on the time of year and harshness of the planting site. The summer drought can make planting in Oregon very challenging for planting after early June. Often trees are pruned to a standard size (e.g., $\sim$30 cm) or transpiration inhibitors applied to the leaves to help them survive in the field. High quality weed control and irrigation are essential to obtain a high rate of survival and growth when using poplar transplants that are actively growing, especially when planting occurs after spring.

3 Management and Its Challenges

At the start of our field research, most management activities were carried out by staff of the Colleges of Forestry or Agriculture as part of the research infrastructure. This setup was commonplace throughout the USA. However, because of rapidly declining support for public agricultural and forest research at OSU and most other academic research universities in the USA, we have had to obtain external research grants to fund, direct, and often personally undertake most of the management activities needed to conduct field research (Fig. 5). This includes obtaining regulatory permits from USDA; basic site preparation and weed control; fencing to exclude deer (>3 m high); planting and fertilization; irrigation management and associated water rights permits; post-planting mowing and weed control; tree harvest and herbicide-assisted devitalization (killing); and site monitoring to kill root sprouts



Fig. 5 Management of field sites. **a** Student Chad Washington planting the ~3 ha third generation sterility trial. **b** Program Manager Kori Ault mowing weeds. **c** Dr. Steve Strauss priming the irrigation pump. **d** Removal of tree roots at the end of a trial for biomass assessment and devitalization

which can appear for several years after harvest and initial attempts to kill trees. Complete killing of all trees, and an absence of root sprouts, is essential for regulatory compliance. Large trees often have extensive root systems that are particularly difficult to completely kill, especially with our model poplar genotypes (which are vigorous natural sprouters). In fact, poplar trees re-sprout so well that they can be coppiced (cut off at ground level) and regrown on a regular basis. While this trait is advantageous for maintaining trees at a smaller size, it is a great nuisance for tree removal. Until there is a complete absence of living sprouts for at least two full years, the field sites remain a regulated piece of land that must be monitored and reported on to the USDA. Individual citizens, not institutions, are the responsible organizations under USDA regulations, making full compliance especially important to the responsible individuals, usually the science director (Strauss).

Any significant deviations from expected conditions in USDA permits must be promptly reported to the USDA. For us, these have included multiple instances where our field sites were partially inundated by the nearby Willamette River after severe winter rain storms, often destroying parts of fences and depositing soil, logs, and other debris onto field sites (Fig. 6).

Weed control is a continual problem and nuisance, and methods for weed suppression such as the use of “shade clothes” around trees (Fig. 4a) have often

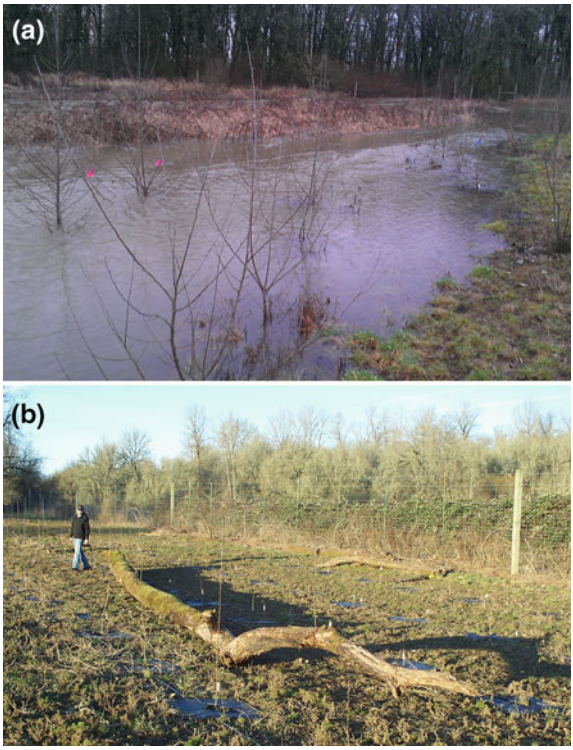

Fig. 6 Field site disturbances. **a** Flooding of a field site by a nearby river, **b** large debris brought in by flood waters. All trees were monitored, accounted for, and the event promptly reported to the USDA

been helpful, though sometimes these provide protection from predators for rodents who can girdle trees. In one field planting during a year of high vole populations, nearly all trees that were protected by shade clothes were girdled, requiring that we abandon and replant the entire experiment (with many hundred trees) the following year when populations had begun to collapse in the area. Weeds can rapidly grow to overtop young trees, especially during our mild and wet winters in Oregon. They also impede access to the trees, making it more difficult to collect samples and data. In addition, weeds provide cover and food for rodents that can damage trees. Mowing provides only a short respite from competition, thus herbicides are often used. However, most herbicides, even when applied with sheltered sprayers, risk damage to poplars even when the trees are dormant. Our experience with weed management has shown us firsthand why herbicide resistant crops are considered of such high value to farmers (who have employed them on a massive scale in agriculture). As discussed below, the superior weed control they afford can significantly increase tree growth.

4 Regulatory Experiences and Shocks

In contrast to many other countries, it is relatively straightforward to get a permit for a field trial of transgenic trees in the USA. One does not need to do extensive research to characterize each insertion and its physiology as is required in some countries (Viswanath et al. 2012); the USDA accepts your claim that it is transgenic then regulates it at a stringency that is proportional to the risk of broad classes of phenotypes, such as if it produces a biopharma product or not, and if it is perennial or not. For multiple year trials of transgenic trees, in recent years the USDA has required full permits rather than the faster and easier "notifications" that were once allowed, thus increasing the burden of permit applications, field trial establishment, and reporting. The USDA is also requiring more stringency in monitoring and reports. Because all transgenic materials must be contained and then removed from the site (and a feasible plan for this must be presented), beyond the broad categories cited above the specific nature of the transgenic trait is not very important when research trials are considered.

For most situations, trees have wild or planted relatives nearby within pollination distance, or have the ability to disperse viable seeds a long distance (e.g., the cottony seeds of poplars, bird dispersed fruits). Thus for most trials trees are not allowed to flower. A benefit of our transgenic poplar models is that they are sexually incompatible with the wild cottonwoods near to our research sites (they are from different sections of the genus *Populus*); this has enabled us to allow flowering in some trials where that trait is important to the research (e.g., to study sterility transgene effects). For many forest trees flowering occurs after the trees are too large to practically remove or bag all flowers (Fig. 1d), which is a major impediment to the conduct of the ecologically and economically most desirable full-rotation trials (Strauss et al. 2010). The USA biotechnology regulatory system is a complex mixture of a trait-risk and method-trigger system that was adapted from prior laws governing plant pests, pesticides, and food safety. In the USDA system, if a plant pest such as *Agrobacterium* is used for gene transfer, or there is any DNA from a plant pest, such as a promoter or terminator, the transgenic plant is putatively a plant pest and assumed potentially harmful until deregulated. Thus, even cisgenic, intragenic, and domesticating traits (Strauss 2003; Bradford et al. 2005) must be fully contained—severely restricting the length and thus the relevance of field trials of transgenic trees. This has made the development of containment mechanisms an extremely high research priority in the USA (Strauss et al. 2009a).

The regulations have resulted in very costly and scary incidences for us. As discussed above, all transgenes produced with a plant pest or DNA from them are presumed to be potentially hazardous, regardless of their real risk or benefit, until "proven" otherwise by a full regulatory petition and review. In today's world where there are groups who will challenge any and all GMO trees in court, this means that USDA must obtain extensive data on all submissions so that they can produce a defensible Environmental Impact Statement. We did not realize how important the distinction between biological hazard and legal burden of proof is until we found that

some poplars in a field trial that flowered abnormally—producing upright catkin-shoot hybrid structures (versus normal drooping catkins), and doing so in the middle of summer (poplars only flower in spring in nature, prior to leaf-out, months earlier; Fig. 7). As required by our permit, we reported this to the USDA, but also indicated that such female catkins in summer—when there is no compatible pollen anywhere—are not a biological hazard. Moreover, all of the trees had genes for semi-dwarfism in them; they would not be competitive with wild trees were any released. We wished to allow them to continue flowering to observe their behavior, and to avoid the significant cost of removal of hundreds of catkins from many dozens of trees. In addition, some of the flowers had unusual transitional phenotypes,

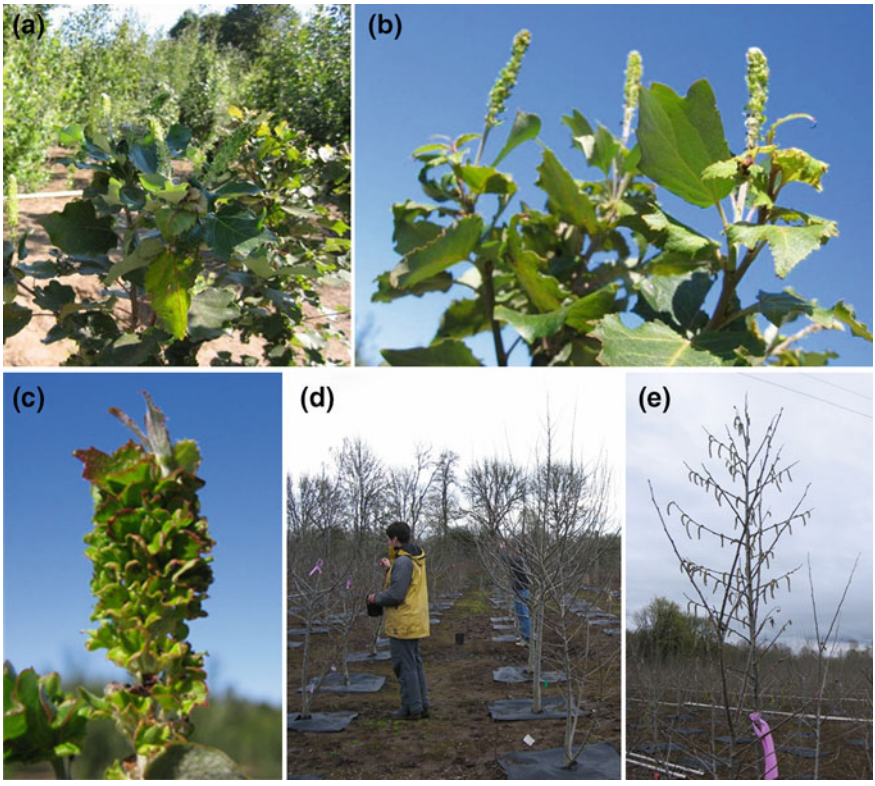

Fig. 7 Collection of unexpected flowers. Trees in one trial flowered unexpectedly in the summer, and our permit requirements (which did not allow for flowering) necessitated removal of every single flower. **a** Trees with fully expanded leaves began to develop flowers. **b** Unusually, flowers grew in an upright conformation instead of the typical hanging orientation. **c** Some flowers had abnormal phenotypes that appeared to be a catkin-vegetative shoot hybrid, with leaf-like organs rather than flowers. **d** Trees flowered again the following spring, again requiring hand-removal of all flowers to comply with permit conditions. The dwarf size of the trees made it feasible to complete the collection without the use of bucket trucks or other machinery. **e** However, trees had numerous catkins, which made the task extremely time-consuming

appearing to be part leaf and part flower. This phenotype was both scientifically interesting and likely to be sterile, further decreasing any possible gene flow. Although the USDA scientists we conferred with agreed with us about the lack of significant risk, and although we had many other trees of the same background genotype for which a permit for flowering on that site had been obtained from USDA, these trees were not intended for flowering thus no such permit had been obtained for them. Finally, after much discussion the USDA scientists felt that they had no choice but to report this as a possible permit violation to their compliance branch (essentially, federal police). Fortunately for us, they informed us of this, and gently but persuasively recommended that we might wish to avoid a legal confrontation over this, with whatever those outcomes might be (e.g., fines, permit revocation, jail time, public embarrassment). Thus we immediately put every student we had available to work in the field removing the catkins before they matured, and reported this action to USDA. We had to undertake a second round of catkin removal the following spring, when these trees flowered at the typical time of year. A possibly cataclysmic legal violation was avoided, one that was prompted by a regulatory system focused on method versus trait, and on legal technicalities vs. science. This is a major reason that we believe that reform of the regulatory system governing field trials is essential if we are to move forward in developing transgenic solutions to speed tree breeding (as we have argued elsewhere; e.g. Strauss et al. 2009b, 2010).

5 Vandalism and Its Impacts

There have been several successful acts of vandalism against transgenic tree research in the USA. The most significant were in 2001 when sites at OSU in Corvallis, the University of Washington in Seattle (UW), and GreenWood Resources (GWR) in Portland, Oregon were attacked. In the latter two cases this caused millions of dollars in damage. The vandalism at our research sites near to Corvallis had very little consequence, as the trees damaged were either non-transgenic, in experiments that were completed, or were recently planted young "seedlings" that easily sprouted after their tops were cut. However, the arson conducted at UW and GWR caused very serious damage, and prompted investments in alarms and magnetic card entry systems at OSU to reduce the risk of similar events. It also caused the movement of our plots to a place where vandalism is likely to have caused lesser damage. Perhaps due to the "9–11" attack on the World Trade Center in New York in fall 2001, the classification of "eco" terrorism as a form of terrorism in the USA (within similar legal consequences), and the FBI's successful pursuit and jailing of many "eco" terrorists, there has been very little further vandalism against biotechnology in the USA, trees or otherwise. Although we have not seen any signs of vandalism since 2001, it remains an ongoing concern, especially as anti-GMO activism seems to be on the rise in the USA.

6 Transformation, Mutation, and Stability

A striking result from our studies of thousands of transgenic poplars is how rarely we observe unintended changes in tree morphology or growth due to the gene transfer and regeneration process, or due to mutagenic effects from where the gene of interest is inserted (Busov et al. 2005a, b). In contrast, there is a great deal of variation in phenotype due to the extensive variation in transgene expression that occurs as a result of the "random" insertion locus and perhaps the unique epigenetic state that is imparted to transgenes with each insertion. We found that dwarf or visible mutants occur at a frequency of around 0.1–1 %. Two rare mutations of note (Fig. 8) were events that did not express their mutations until after dormancy and resumption of growth in the field (they had been planted after continuous in vitro propagation following transformation)—suggesting that dormancy might have triggered epigenetic changes in gene expression due to a somaclonal mutation. One mutant showed much reduced field growth and another both reduced growth and mottling of leaves (Ault et al. 2016). However, for most transgenic poplar the process of producing transgenic poplars does not seem to impact their growth. In a recently published study, we compared the growth of transgenic poplars containing various GUS reporter constructs to wild type controls after 3 years in the field; there were no significant or even modest differences in growth rate (Elorriaga et al. 2014). Although it is difficult to compare species that are grown and transformed differently, and have distinctive morphology, it is our impression that poplars suffer an unusually low rate of somaclonal and transformation-related variation compared to many other species.

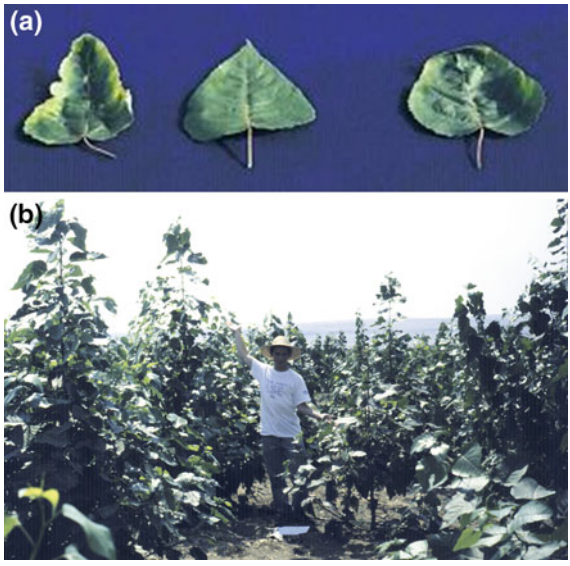

Fig. 8 Rare tree abnormalities observed in transformation events with hybrid cottonwoods. **a** Leaves with variable shapes and mottling (Ault et al. 2016: control in *center*), and **b** a dwarfed mutant (*right*) compared to majority of transgenic trees (*left* and rest of plantation)

We have tested hundreds of events in field trials that showed a visibly detectable phenotype, mostly herbicide resistance or insect resistance under insect pressure. We have not observed any cases of gene silencing over years that resulted in a sudden loss of phenotype. Li et al. (2009) showed that herbicide resistance was stable over 8 years and multiple coppicing (cutting and regrowth), and Klocko et al. (2014a) showed that *Bt* gene expression was stable over 14 years despite multiple coppice cycles. Likewise, the early flowering observed by Mohamed et al. (2010) due to RNAi suppression was stable over multiple years, as was RNAi against an herbicide resistance gene (based on qRT-PCR; Li et al. 2009) Reporter gene expression has also been remarkably stable over time in our multi-year field studies (Meilan et al. 2001; Li et al. 2009). Male-sterility due to tapetal ablation was very high and essentially complete over 4 years in the field (Elorriaga et al. 2014). It appears that poplar, either because of its low rate of somaclonal mutation or because (in contrast to annual species) it is not subjected to rounds of sexual propagation after transformation (meiosis and related processes might trigger gene silencing at a higher rate than vegetative development), has a very low rate of instability in gene expression.

7 Biological Lessons

We have conducted a number of field studies with the goal of testing biological hypotheses under conditions of physiological relevance to trees, or ascertaining if transgenic modifications could provide value in a plantation forestry context. The results of these studies are mostly published; we highlight a few below.

7.1 Acceleration of Flowering

We have found that reducing the expression of a poplar homolog of the *Terminal Flower 1* gene using RNAi gave rise to trees of normal form and growth rate, but which flowered 2–4 years earlier than normal (Mohamed et al. 2010). As a dominant gene it could therefore be used to accelerate flowering in poplar breeding programs, then segregated away in progeny during further selection and propagation. In contrast, overexpression of poplar Flowering Locus T generally does not lead to viable pollen and seed (but see Hoenicka et al. 2014) and causes large pleiotropic effects that would impair growth and survival in the field (Zhang et al. 2010). Many other early flowering transgenes that are effective in *Arabidopsis* do not function at all in transgenic poplar (Rottmann et al. 2000; Strauss et al. 2004).

7.2 Sexual Sterility

We have found that a male-sterility transgene functions well in poplar over four growing seasons, preventing virtually all pollen production (Elorriaga et al. 2014). In addition, by field testing and comparison we also found that the barnase gene we employed impaired the growth rate of nearly all transgenic lines, stimulating the development of attenuated forms of barnase in subsequent commercial constructs used in pine and eucalypts (Zhang et al. 2014). Recently, Klocko et al (2014b), reported that RNAi suppression of the poplar *LEAFY* gene prevented catkin maturation while allowing normal vegetative growth. It was shown in a female genotype, but because of the function of *LEAFY* is expected to also work in male genotypes, giving complete sexual sterility in a field-grown tree for the first time. In a greenhouse experiment, a barnase construct controlled by the poplar *LEAFY* promoter and attenuated to various degrees by its specific inhibitor barstar gave normal growth and development of poplar in the greenhouse. However, in the field many of the same trees expressed diverse malformations and much slowed growth, providing a vivid demonstration of the importance of field testing to observe pleiotropic effects (Wei et al. 2006).

7.3 Gene Tagging

Although great strides have been made in recent years in high precision genomic mapping, it remains very difficult to definitively link allelic variants within tree populations to specific quantitative traits. Such linkages were essentially impossible to make at the time we began work on gene tagging in about 1995. The goal of our work in gene tagging was to create allelic variants from natural genes that were large and strong enough to be definitively associated with the affected trait, and to be expressed when hemizygous as primary transgenic plants (i.e., genetically dominant, as poplars cannot be selfed to produce loss of function homozygotes, and inbreeding depression in trees creates additional confounding phenotypic variation). Activation tagging, a recent innovation, seems to be an answer (reviewed in Busov et al. 2005a, b). In this method, a strong enhancer is ~randomly inserted into the genome such that, when it lands near enough to a gene to affect its promoter and cause abnormal upregulation of the gene, it is easily identified by methods such as inverse- or TAIL-PCR (reviewed in Busov et al. 2010a, b). This approach has been used to identify several genes whose functions in tree biology were previously unknown or poorly understood (e.g., Busov et al. 2003; Yordanov et al. 2014). One problem is that the large majority of activation-tagged transgenic trees does not show visible trait modifications in the laboratory or greenhouse, and many of those seen, such as modified leaf morphology, are not of particular interest to tree biologists. We therefore created two hectare-scale field trials of our activation-tagged populations (Fig. 2), which we showed led to the identification of many more, and

more biologically interesting, trait modifications than had been observed in the greenhouse alone (Busov et al. 2010a, b). The modified traits included timing of bud flush and bud set, timing of leaf senescence, bark morphology, wood color, crown form, wood chemistry, phototropism, and leaf pubescence (examples in Fig. 2). After the affected genes are identified, RNAi is typically used to understand the gene's role in the absence of overexpression (e.g., Yordanov et al. 2014).

7.4 Form and Architecture Modification

Genetic modification of gibberellin (GA) synthesis and signaling have been used extensively in agriculture to produce semi-dwarf plants and are the basis of the green revolution that revolutionized cereal yields in many parts of the world. With the identification of the underlying genes, we used transgenic methods to study whether similar modifications would be effective in trees, and if they are useful for trees grown as ornamentals (where dwarfism is often highly desirable) or for bioenergy or short rotation forestry (where semi-dwarfism could improve wind-firmness, wood quality, and stress resistance: (Klocko et al. 2013). The transgenes produced a burst of morphological variability in leaf size and color, stature, and crown structure that would be impossible to fully appreciate without growth in the field (Zawaski et al. 2011). This method could clearly be of great value for ornamental horticulture if GMO trees were not so stigmatized and costly to market (Etherington et al. 2007). We also conducted high density field trials at different spacings to test if, as with agricultural crops, the relative performance of semi-dwarfs was most expressed at high planting density, and if semi-dwarfs would have a significant disadvantage in competition with wild type (dwarfism genes have been suggested as a means to mitigate ecological impacts from transgene release). Both hypotheses were supported by the field results, and the semi-dwarf poplars shown to have much higher allocation of biomass to roots compared to shoots, suggesting that they could promote drought tolerance and wind-firmness (Elias et al. 2012). Finally, we have studied the potential for overexpression of genes that induce synthesis of active GAs to improve growth rate in the greenhouse and the field. The transgenes were very successful in many experiments, including in modifying the allocation of biomass among plant organs, but growth rate improvement was highly variable and the correlation between field and greenhouse growth extremely poor (Viswanath et al. 2011). Were the trees not transgenic, the next step would have been much expanded field plantings, similar to that of a conventional breeding program. However, we have been unable to obtain funds for such work, mainly because of the national disinvestment in transgenic technology by grant agencies.

7.5 Ecophysiology and Lignin Perturbation

In the 1990s and early 2000s, the benefits of transgenic lignin modification for improved growth rate and improved pulp or biofuel yields had been widely touted. Other than some high quality field trials in Europe, many of these claims were based on poorly designed greenhouse or field evaluations, or lacked field data entirely. One widely acclaimed transgenic modification was *4CL* downregulation (Hu et al. 1999). To see if these benefits were real, we produced transgenic poplars with an antisense *4CL* gene provided by the Chiang laboratory at North Carolina State University and grew them in the field in Oregon for two years. In contrast to expectations based on casual observation or greenhouse experiments, the growth and drought tolerance of those trees in the field were disappointing; the traits of interest were either unaffected or impaired, and there was no benefit for bioethanol production. Instead, the most strongly downregulated trees had badly malfunctioning and collapsed xylem cells which made the wood stiffer and less able to transport water (Voelker et al. 2010, 2011a, b). This case is perhaps the most striking demonstrations of the need, early in research, for field trials over several years, with different genotypes, and at multiple sites when transgenes are used to significantly modify fundamental aspects of plant structure and physiology.

Economic value. After promising results in obtaining highly pest or herbicide tolerant trees in field screens (Meilan et al. 2000a, b) we worked with industry partners to establish "management trials" on their land. In these trials, large numbers of trees were planted and managed under near-operational conditions. In the case of insect resistance, we used a modified gene from *Bacillus thuringiensis* (a variant of Bt cry3a) provided by Mycogen that we had shown earlier should produce a toxin that is lethal to a major pest of poplar, the cottonwood leaf beetle (*Chrysomela*) (James et al. 1998). The results of the screening and growth trial revealed a very strong and stable benefit from the expression of the gene that—even under conditions of low insect pressure (damage that was not visually obvious in most trees)—led to a 10–20 % improvement of volume growth over 2 years in the field (Klocko et al. 2014a). In a management trial of highly glyphosate tolerant trees, (Ault et al. 2016) found that in a weed control regime designed to take advantage of herbicide tolerance, weed populations were greatly reduced in density and tree volume growth was increased by approximately 20 % over two growing seasons. There are likely to be underestimates of the benefits that would accrue when pest damage is high, and when weed management has been more fully adapted to take advantage of herbicide resistance. These results suggest that, if combined with a genetic containment option to reduce management trade-offs and facilitate regulatory and social approval, these traits could be of considerable value in the management of short rotation poplar plantations.

8 Regulatory Lessons and Conclusions

As biotechnologists, there is nothing more gratifying than seeing a transgenic concept based on biological research in a model organism, or when following upon a lab or greenhouse experiment, express itself in the field. It is also instructive when a trait does not show what you expected, as it teaches you about how traits depend intimately on the ecophysiology of the organism—which is influenced by many factors. These include species and genotype; transgene expression intensity and pattern; stage of tree development; and environment. The unpredicted outcomes become probes of specific developmental and physiological processes. To produce a useful modified organism with complex traits requires plant breeding-scale studies in many genotypes and environments, over many years of growth. However, these are rarely done in public sector research due to high costs and regulatory risks or preclusions. As a consequence, there is a dearth of information on long-term performance of transgenic trees in the scientific record (private sector studies are rarely and selectively published). Old myths, including of the unworkable instability, dramatic danger, or magical performance of transgenic trees persist much longer than necessary as a consequence.

Our experience has shown that the transgenic trees we have worked with–mainly poplars, eucalypts, and sweetgum–perform reliably and stably. Unfortunately, the most valuable traits for production and tolerance of environmental stresses, especially for trees under climate associated stresses, are likely to be those that modify physiology, including for pest resistance. Thus, as discussed above, field studies in many genotypes and environments are needed. Unfortunately, under current process-based regulatory regimes (which implicitly assume all transgenic trees are hazardous), it is nearly impossible to conduct the kind of wide-ranging trials needed to test these genes and incorporate them into breeding programs (Strauss et al. 2010, 2015). Such regulations would seem to be a violation of the Precautionary Principle, if you believe, as we do, that it is foolish not to develop transgenic options for coping with growing stresses on forest and agricultural systems. Our experience has suggested that transgenic trees are a valued and reliable tool that could, if unlocked from its strong regulatory and market restraints in most countries, make large and extensive contributions. But fundamental regulatory reforms will be needed if the required biology and breeding studies, in the field, are to occur beyond that in a very few companies, academic laboratories, and countries. Given the extensive political resistance to all kinds of GMOs, this will take leadership, communication, and investment at the highest levels of government, business, and civil society.

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Box 1 Contents snapshot for MIAPE-GI

The full MIAPE-GI document is divided into two parts: an introduction providing background and overview of the content and a full list of the items to be reported. The guidelines have been designed to cope with different types of workflows, as performed by particular software packages. As such, a number of items are optional if they refer to a specific procedure not employed by the software used. The MIAPE-GI guidelines themselves are subdivided as follows:

- General features describing the type of electrophoresis performe for analysis and the analysis software used.
- The gel analysis design with respect to replicates, groupings and
- Image preparation steps before bioinformatics analysis such as sc
- Image processing, such as image alignment performed by bioinforma
- Data extraction, including feature detection, feature matching a quantification (if performed).
- Data analyses performed, for example, extracting features with sig expression.
- Results of data analysis, including feature vocabulary, matches and where appropriate.

versions or by complementary modules, such as MIAPE-GE, which can be obtained from the MIAPE web page (http://www.psidev.info/miape/). As is the case for all MIAPE modules, this specification does not recommend a particular format in which to transfer data nor the structure of any related repository or document.

These guidelines will evolve as circumstance dictates. The most recent version of MIAPE-GI is available from the HUPO-PSI website and the content is replicated here in **Supplementary Table 1**. To contribute or to track progress to remain 'MIAPE compliant', browse the HUPO-PSI website (http://www.psidev.info/miape/).

Note: Supplementary information is available on the Nature Biotechnology website.

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The 20-year environmental safety record of GM trees

To the Editor:

In a commentary last May, Strauss *et al.*[1] pointed out that opposition to genetically modified (GM) organisms has recently intensified on GM trees and that recommendations of the Conference of the Parties (COP) to the Convention on Biological Diversity (CBD) have encouraged regulatory impediments to undertaking field research. We concur with Strauss *et al.* that the CBD appears to be increasingly targeted by activist groups whose opinions are in stark contrast to the scientific consensus and indeed the opinions of most respected scientific and environmental organizations worldwide. Strauss *et al.* call for more science-based (case-by-case) evaluation of the value and environmental safety of GM trees, which requires field trials. However, the regulatory impediments being erected by governments around the world, with full corroboration of the COP, are making such testing so costly and Byzantine, it is now almost impossible to undertake field trials on GM trees in most countries. Here we summarize the key published evidence relating to the main environmental concerns surrounding the release of GM trees (**Box 1**). On the basis of our findings, we urge the COP to consider the opportunity costs for environmental and social benefits, and not just risks, in its deliberations of field trials and releases.



A very large amount of performance and safety data related to GM crops and trees has now been gathered since field trials were first initiated in 1988 (ref. 2). Our search in publicly accessible databases worldwide

Box 1 Commercially successful GM trees

Few GM tree species have as yet been deployed commercially. Two notable exceptions are the following: *Bacillus thuringiensis* toxin (*Bt*)-expressing poplar trees in China; and papaya trees expressing the viral coat protein gene of papaya ringspot virus (PRSV) in Hawaii.

Approximately 1.4 million *Bt* poplars have been planted in China on an area of ~300–500 hectares along with conventionally bred varieties to provide refugia to avoid the development of *Bt* resistance in insects. The trees are grown in an area where economic deployment of poplar was previously impossible due to high insect pressure. GM trees have been successfully established and have successfully resisted insect attack. The oldest trees in the field are now 15 years old (Minsheng Wang, personal communication). No harm to the environment has been reported.

Experiences with GM papaya trees also illustrate multiple benefits[15]. The Hawaiian papaya industry faced serious threats in 1992 when PRSV was detected in plantations, and production dropped from 55 million pounds to 26 million pounds in 1998. In 2001, 3 years after the release of PRSV-resistant GM papaya plants, production was up to 40 million pounds. As an additional benefit, the GM papaya actually enabled the economic production of non-GM papaya in the same area because the GM trees kept infestation rates in the area well below economically problematic levels.

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reveals >700 field trials with GM trees (including forest trees, fruit trees and woody perennials). None of them has reported any substantive harm to biodiversity, human health or the environment. In the following paragraphs, we summarize our main findings as they relate to ecological impact, the stability of transgene expression over time, the effectiveness of transgene containment and the status of nontarget organisms on leaves, stem and in soil.

Field trials with GM poplars (*Populus* sp.) with modified lignin composition were among the first to include potential ecological impacts on the environment as goals. In this case, the poplars were engineered to express antisense transgenes that reduced the expression of lignin biosynthesis genes cinnamyl alcohol dehydrogenase or caffeic acid/5-hydroxyferulic acid *O*-methyltransferase. Field trials of these trees, conducted in the UK[3,4], were regularly inspected for alterations in growth and development, as well as for damage caused by insects, including ladybirds, ants, aphids, copper beetles, earwigs, shield bugs, froghoppers, caterpillars, spiders and fungi. No differences were observed comparing the wild-type and GM trees[3,4]. In addition, after termination of two trials in the UK and France[5], analysis of the levels of carbon, nitrogen and microbial biomass as well as of the soil microbial population revealed no consistent differences between plots with wild-type trees and plots with GM trees. In fact, the only significant differences in these parameters were observed between the soil of the field trial and the soil taken under the grass just <4 meters away from the field trial, indicating that the influence of the different vegetation types is considerably larger than the variation induced by the genetic modification[5]. Although decomposition assays revealed that the roots of lignin-modified trees do compost slightly faster than those of wild-type trees, this is in agreement with the role of lignin in resistance to biodegradation and the expectations of the researchers. Thus, no unexpected ecological impacts could be attributed to the GM trees; instead, differences in soil characteristics and microbial biomass were caused by environmental variation[4,5].

With relation to the ability of transgenes to be stably expressed over many years, studies of GM poplars over 3 to 8 years have found no evidence for loss of transgene expression in the field[6–8]. Nearly all of the instability observed is during *in vitro* production and propagation. This suggests that once GM trees pass *in vitro* and early field screens for stability, their traits remain highly stable[6–8]. The same also applies to RNA interference (RNAi)-based gene suppression traits, which have been shown to be stable and reproducible over multiple years and independent of outdoor temperature. This suggests that gene suppression continues to occur, even during early bud development and leaf senescence[9]. In two other field trials poplar trees resistant to glufosinate by virtue of expression of the phosphinothricin acetyltransferase[10] or poplars carrying the *Agrobacterium rhizogenes rolC* gene have also been confirmed to stably express the transgenes after selection of GM lines[10,11]. Unpublished data from trials in New Zealand of transgenic pine, which express

the antibiotic resistance gene encoding neomycin phosphotransferase (*nptII*), also provide further corroboration of transgene stability in the field (C. Walter, unpublished data).

Transgenic containment traits for mitigating gene flow have also been effective when tested in the field. Studies with male transgenic poplar trees containing a male-sterility gene showed that several transgenic events had very low or undetectable levels of pollen production, which persisted over several years[12]. This suggests that containment genes can be highly efficient and stable. The Strauss laboratory has produced ~1,000 transgenic events in poplar with advanced forms of sterility genes (via RNAi or dominant-negative mutations) with the intent of similar field studies.

Finally, data on the status of nontarget organisms on leaves, stem and in the soil surrounding GM trees also indicate that the traits tested thus far are comparable to wild type. A comparison of wild-type and *rolC* transgenic poplars in German field trials revealed no differences in the status of nontarget phytopathogenic fungi on leaves and stems, or evidence of differences in carbohydrate and hormonal metabolism in the transgenic trees (M. Fladung, unpublished data). These studies have also systematically investigated the possibility of horizontal gene transfer to mycorrhizal fungi. Transgenic poplars carrying a fungal-specific promoter controlling the *Streptococcus hygroscopicus bar* gene were planted in the field to assess if horizontal gene transfer to the mycorrhizal fungi living in association with the transgenic trees occurred. Subsequently, large screening programs were initiated to identify putative phosphinothricin herbicide (Basta)-resistant mycorrhizal fungi. Although the results remain unpublished, the investigators running the trials have communicated that even though >100,000 mycorrhizal fungi were isolated from roots of the transgenic trees, there was no indication of a horizontal gene transfer event[10] (M. Fladung and U. Nehls, unpublished data).

Nontarget effects have also been studied in transgenic pines. In experiments conducted in New Zealand using radiata pine (*Pinus radiata*) genetically modified with *npt*II and genes related to reproductive development, the impacts on invertebrates and soil microbial populations were assessed over a period of 2 years (on trees that had been grown in the field for up to 9 years; personal communication). When the composition and abundance of invertebrate populations usually present on non-GM radiata pine were compared with those on GM pines, no differences were found other than seasonal differences, and invertebrate species and numbers were unchanged[13]. Feeding studies with GM needles revealed no impact of transgenic material on fertility or fecundity of the invertebrates. Microbial populations living in association with, or close to, the roots of trees were characterized using an approach capturing the culturable and nonculturable fractions of microbes. Although seasonal differences were observed in population structures, no significant differences between GM and unmodified trees were found (C. Walter, unpublished data). These experiments again show that variation caused by environmental factors is much more pronounced than variation induced by the genetic modifications studied.

Decisions on whether or not to use GM (or conventionally bred) organisms should be based on a scientific evaluation of possible risks associated with a particular new trait and the degree of novelty of the genes encoding it. However, it is also important to keep in mind the significant environmental benefits that such organisms could provide. The negative effects of the creeping regulatory burdens are becoming progressively more obvious as GM methods cannot be effectively employed despite the growing anthropogenic threats to native forests, the urgent needs for new biofuels and biomaterials, the already substantial impacts of climate change on forest health and the growing demand for forest products[14,15]. And all of this in the face of pressing demands for increased forest conservation. Given these grave challenges, among which are serious threats to the very survival and basic productivity of native and planted forests, we need to put hypothetical residual risks of GM in context. In our view, they appear very modest indeed.

Sooner or later, the COP should recognize the huge opportunity costs its current recommendations impose for GM technology. When it meets in Nagoya, Japan, in October, COP should urgently take note of the scientific evidence on the biosafety of GM traits that have been tested in the field so far and reconsider the regulatory and political hurdles that currently make meaningful field tests of GM trees almost impossible. The strong concerns against all GM plants and trees, initially expressed more than 20 years ago, are no longer justified. They are obviated by the long record of safety obtained from hundreds of field trials with several transgenic traits and the urgent societal and environmental problems for which the technology could be one additional, valuable tool. Therefore, we recommend the COP seriously consider the endorsement of policies that actively promote, rather than retard, further field testing of GM trees.

COMPETING INTERESTS STATEMENT
The authors declare that they have no financial competing interests.

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Viewpoints

Social and biological innovations are essential to deliver transformative forest biotechnologies

Summary

Forests make immense contributions to societies in the form of ecological services and sustainable industrial products. However, they face major challenges to their viability and economic use due to climate change and growing biotic and economic threats, for which recombinant DNA (rDNA) technology can sometimes provide solutions. But the application of rDNA technologies to forest trees faces major social and biological obstacles that make its societal acceptance a 'wicked' problem without straightforward solutions. We discuss the nature of these problems, and the social and biological innovations that we consider essential for progress. As case studies of biological challenges, we focus on studies of modifications in wood chemistry and transformation efficiency. We call for major innovations in regulations, and the dissolution of method-based market barriers, that together could lead to greater research investments, enable wide use of field studies, and facilitate the integration of rDNA-modified trees into conventional breeding programs. Without near-term adoption of such innovations, rDNA-based solutions will be largely unavailable to help forests adapt to the growing stresses from climate change and the proliferation of forest pests, nor will they be available to provide economic and environmental benefits from expanded use of wood and related bioproducts as part of an expanding bioeconomy.

With the recent launch of the new section of *New Phytologist* on 'transformative plant biotechnology (Halpin *et al.*, 2023)', it is an apt time to consider what is needed for transformative leaps that can enhance forest tree biotechnology. By biotechnology, we mean modification by recombinant DNA (rDNA) in the form of gene-editing (i.e. targeted modification of native DNA) or gene insertion, not applications such as genomic selection that can accelerate conventional breeding. We are both biotechnologists who have conducted basic and applied research on genomic and molecular biology of trees, including field trials of prototype-modified trees in the United States and Europe (e.g. reviewed in Pilate *et al.*, 2002; Strauss *et al.*, 2016; Chanoca *et al.*, 2019; De Meester *et al.*, 2022b). We have experience in research, translation, and interaction with legal and market systems, as well as engagement with the general public.

We believe there are urgent needs for both social and biological innovations if biotechnology is to have a significant and beneficial impact on the productivity and quality of tree plantations or other types of forests. First, we need to create a regulatory and market environment that is conducive to innovation, rather than one that poses tremendous impediments; the latter is the rule throughout most of the world today. This is especially critical in the context of today's reality of rapid climate change, where genetic improvements of all sorts must occur as rapidly as possible if they are to help the ongoing adaptation of tree plantations and other forests to changing climates and pathogen pressures (Jacobs *et al.*, 2023). Second, there is a critical need for accelerated research, both fundamental studies such as the analysis of basic processes underlying plant regeneration and wood development, and technology advancements to make genetic modification more efficient and reliable in multiple tree species and genotypes. Third, it is essential that rDNA methods are well-integrated with conventional and genomic breeding, though many technical and social obstacles make this difficult. This would include extensive field research, as most results from laboratory or glasshouse experiments have limited relevance to field environments due to the very different stresses and other aspects of tree ecophysiology in the field vs indoor conditions (Viswanath *et al.*, 2012; Khaipho-Burch *et al.*, 2023). Field research is also important for public education about the true benefits and risks of modified trees. Finally, there are a number of larger contextual issues related to rDNA-modified forests, including questions of how to evaluate risks from rDNA technology vs that from other forms of breeding and management in a climate change world, and when and where rDNA methods should be applied. We believe that the primary uses will be in intensively managed, short-rotation clonal plantations, for which wood products have distinct industrial uses, but that there are also important opportunities to help with the protection or restoration of wild or feral forests. We will discuss each of these areas, giving examples from our own research and experience.

Social innovation

Perhaps the most impactful social constraint is the implicit or explicit legal presumption of guilt due to the use of rDNA methods, with only minor exceptions for gene-edited plants that are similar or identical to those that could be produced from conventional breeding (Strauss *et al.*, 2010, 2022). By contrast, conventional breeding methods – whether they involve species transfer among continents, production of novel hybrids, clonal deployment, or radiation/chemical mutagenesis – have no such presumption of guilt. Some exceptions exist in the form of strict regulations on some exotic species that are known to be highly invasive and

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damaging. This dichotomy is true essentially everywhere, including in the United States, where it is inherent to both the US Department of Agriculture (USDA) and Environmental Protection Agency (EPA) regulatory systems. However, the recently enacted USDA SECURE regulatory system is a modest exception; though regulation is triggered by the use of rDNA methods, regulatory decisions are intended to focus exclusively on whether there is a plausible risk that an rDNA-modified plant will cause or exacerbate a pest problem. It also has a means for rapid deregulation decisions, and exemptions for simple types of gene-edited plants. We regard this system, with its trait focus and options for exemptions and rapid decisions, as a first step in the right direction (Goralogia *et al.*, 2021). There are now also regulatory exemptions for simple gene-edits in the regulatory systems of many other countries around the globe (Smyth, 2019; Turnbull *et al.*, 2021).

We are also encouraged by the mostly growing acceptance of rDNA-based methods by the public. The 'eco'vandalism of recent decades against rDNA trees, whether due to concerns about violation of trees that are perceived as 'natural' or concerns about corporate control of forests, appears to have abated internationally. In addition, the sensational and highly derogatory headlines about rDNA crops, particularly in Europe, have become extremely rare. Although concerns about the safety of 'GMO food' have risen in the United States in the last decade and are shared by approximately half of the public, many view GMOs as a 'necessary evil' given their perceived value for helping to 'feed the world' amidst growth in population and standards-of-living (Funk, 2020). Likewise, social media messages have become distinctly less negative in recent years (Evanega *et al.*, 2022). Unfortunately, scientifically false statements, mostly negative ones, are still very common in mainstream print and online media, with a rate of 9% and considerably higher in Africa (*c.* 20%) during 2019–2021 (Lynas *et al.*, 2022).

On the other hand, the US EPA continues to increase its regulatory coverage in what seems far beyond its original mandate to protect the public and environment from pesticide harms; it now covers growth-improved plants and pest-resistant plants that do not involve pesticide use at all, but engage natural resistance mechanisms. This is a very effective deterrent for the use of pest-resistant trees, and indeed even the blight-resistant American chestnut, which involves no novel pesticides, has been held up in regulatory limbo there for years. The US EPA's 'exemption' for gene-editing is also very limited, still requiring safety data and proof that the edit is already present in a sexually compatible relative (Stokstad, 2023). It appears that congressional action will be needed if this regulatory overreach is to be reversed. A similar sentiment was reflected in a recent US Senate report; it highlighted the need for regulatory discipline if biotechnology innovations are to flourish (National Security Commission on Emerging Biotechnology, 2023).

In contrast to most of the regulatory systems world-wide, for decades, scientists have called for a science-based regulatory system that focuses on the comparative risk associated with the presence vs non-presence of the trait, not with the method employed, and that would allow a broad array of rDNA-modified varieties to be planted and commercialized unless there are tangible, major risks associated with the novel trait (e.g. National Academies of Sciences Engineering and Medicine, 2016). Such risks could include novel disease resistance mechanisms that might substantially impact wild or feral populations, or the generation of herbicide-resistant plants that will compromise plantation or weed management. A system focused on trait-based risk would also not preclude all gene flow during field research and breeding, thus allowing rDNA-modified plants to be readily deployed alongside conventionally bred plants during field trials unless they pose a tangible risk at the relevant rates of gene flow (normally very low in research/breeding trials) compared with potential benefits; this is critical for the expanded use of field trials as discussed below.

We note that a change to a trait-based system would not imply that all gene flow is risk-free, only that society will tolerate low levels and their impacts, just as it does for conventional breeding and agricultural production of the large majority of crop species, in exchange for the benefit of accelerated innovation to help cope with climate change and social stresses on food and fiber production systems. Of course, regulations that define what 'tangible risk at the relevant rates of gene flow compared to potential benefits' would look like is complex and beyond the scope of this paper to explore in depth; but, we imagine a system similar to the Canadian rDNA regulatory system (Lassoued *et al.*, 2020) in that it would apply to both rDNA and conventional breeding. The new USDA SECURE system, which tries to define 'plausible plant pest risk', faces similar challenges in definition (no surprise given biological and ecological complexity and diversity). A biological framework for such a system, which might be based on changes in native genes and their expression as familiar vs modifications that impart truly novel and exogenous trait mechanisms (e.g. new pest resistance toxins), and whether the trait is a domesticating one or likely to exacerbate weediness, was described more than two decades ago (Strauss, 2003). Due to extensive global trade in food and forest products, such a change to a trait-based system would ultimately need to occur at an international level, presumably through modifications to the Cartagena Protocol. Thus, it will require strong international leadership at the highest political levels.

In addition to regulatory considerations, the market also provides major constraints to rDNA innovation. Most importantly, the now widespread forest certification systems impose a complete ban on the use of rDNA-modified trees in production forests (Strauss *et al.*, 2019). This ban has been long-standing, and recent efforts by the Forest Stewardship Council (FSC) to lift the ban, or to at least allow joint research projects with developers of rDNA-modified trees, have failed due to its controversial nature within the FSC. This ban has discouraged investment in biotechnology throughout most of the globe – as our personal experience working with forestry companies has clearly shown. Regulatory revisions notwithstanding, until such bans are broadly and permanently lifted, most companies will continue to be unwilling to undertake significant investments in rDNA biotechnology.

Science and technology innovation

Informed by genomic databases and a growing understanding of the genes and pathways that control plant physiology and growth, the opportunities and the efficiency of trait modification have

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expanded rapidly. Innovations such as basic gene transfer and regeneration methods, which were first developed in model plants (Barton *et al.*, 1983; De Block *et al.*, 1984) and then applied to crop plants, found their way into forest trees very soon after, first in the model tree poplar (Fillatti *et al.*, 1987) and then in horticultural tree crops, such as apple (James *et al.*, 1989), followed by the more difficult to transform conifers (Ellis *et al.*, 1989). Gene silencing (or RNA interference (RNAi)) was first observed in the model plant petunia (Napoli *et al.*, 1990), and once its mechanism was revealed and its application was optimized, it was widely applied to modify a diversity of traits in forest trees, from flowering (Rottmann *et al.*, 2000) to wood chemistry (Van Doorsselaere *et al.*, 1995). Gene-editing methods using for instance zinc fingers were rapidly applied to forest trees (Lu *et al.*, 2016), and soon replaced by CRISPR, which has ever since been widely used to study tree physiology, flowering, and wood chemistry (Fan *et al.*, 2015; Zhou *et al.*, 2015; Elorriaga *et al.*, 2018; Sulis *et al.*, 2023). It is clear that the power and precision of rDNA methods will continue to grow, providing a further motivation for breeders to start taking advantage of its capabilities. We describe two areas of economic importance, in which rDNA innovations have been growing, as examples.

Regeneration and transformation

Large improvements in transformation efficiency are essential for biotechnology to address the wide diversity of germplasm in tree breeding programs. In crop plants, genetic transformation methods have taken great steps forward in the last decade, largely the result of the use of developmental or morphogenic regulator (*DEV*) genes such as *WUSCHEL* and *BABY BOOM*. The benefits have been the greatest in monocots (cereal crops), which, combined with decades of research to develop embryogenic propagation systems, have led to transformation protocols that work in a wide variety of species and explant types (Gordon-Kamm *et al.*, 2019; Wang *et al.*, 2023). Similar progress is now being made for dicot species, including poplar, though these appear to often require different types of *DEV* genes or delivery systems that fit with their biology. For example, Ryan (2022) reported that the success of using the recently popular GRF-GIF types of *DEV* genes in poplar and eucalypts was highly genotype- and construct-dependent, requiring substantial gene customization and refinement of transformation protocols for successful use. Many woody species, both dicots and most notably conifers, also have distinct reproductive biology and/or regeneration systems compared with commonly studied, seed-propagated annual plants. In addition, the epigenetically 'mature' clonal tissues usually employed have distinct and highly variable levels of totipotency, which appear to affect their potential for embryogenesis or organogenesis. As discussed earlier (Strauss *et al.*, 2022), transformation and gene-editing systems for forest trees will require substantial further innovation and refinement for *DEV* genes to help mitigate the transformation bottleneck.

The very limited budgets for research in forest tree regeneration and transformation have prompted most researchers to simply try the genes and *Agrobacterium* strains available from agricultural crops – rather than to seek to understand the fundamental causes of

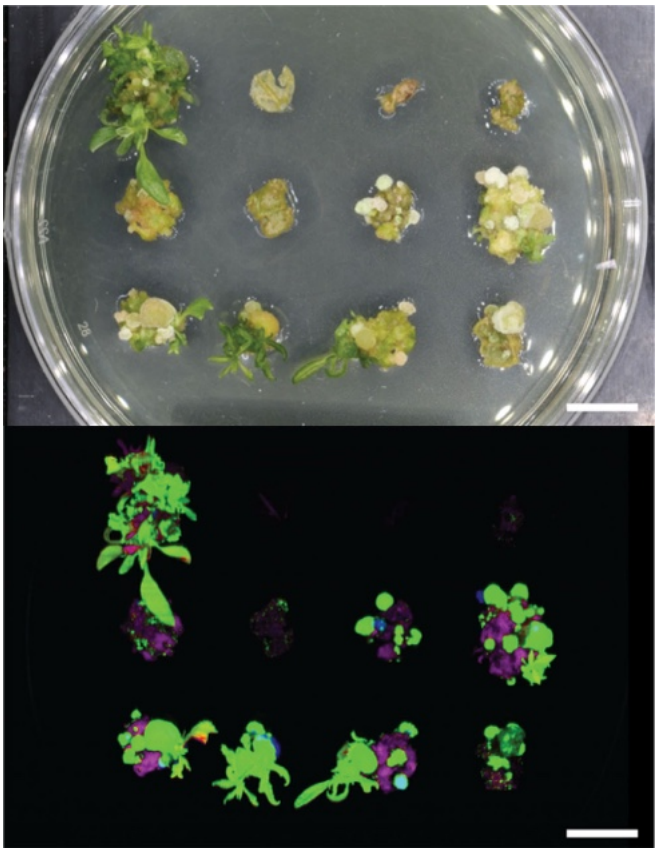

Fig. 1 New phenomic systems enable more precise and rapid means for scientific research and technology development. Here, new imaging and machine vision methods facilitated genome wide association studies (GWAS) of transformation. A hyperspectral imaging system, combined with machine learning, was used to quantify transformation rate as part of a GWAS of its biological basis in *Populus trichocarpa* (Nagle *et al.*, 2024a). Brightfield image above, and false color image below showing *GFP*-expressing tissues in green, and non-transgenic, chlorophyll-containing tissues in magenta. Bars: 1 cm.

recalcitrance to standard transformation methods. However, it is likely that significant inroads could be made by taking a fresh look at the distinct biology of trees and their development first. For example, recent genome wide association studies (GWAS) facilitated by new phenomic methods (Fig. 1) have shown that there are numerous genes involved with amenability to regeneration or transformation, and very few of these appear to be identical to those that have been effective in aiding transformation in crops (Nagle *et al.*, 2023, 2024a, b). In addition, the massive immune response, extreme genetic diversity, and strong epigenetic/ontogenetic variation discussed above warrant investigation into their biological mechanisms and variability. Finally, some of the basic technologies required for advanced rDNA-based modification, such as that of gene excision, appear to work very poorly or not at all in trees, likely a reflection of the extensive DNA methylation that accompanies recombinase induction (Liu *et al.*, 2021). The desire to remove any transformation-promoting genes from the genome to avoid pleiotropy, or to remove gene-editing reagents to ease regulatory acceptance and reduce off-target mutagenesis, will require smarter excision systems as well as much improved transformation

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technologies. There are also promising editing systems that rely solely on transient expression or viral inoculation (e.g. Huang *et al.*, 2023; Sun *et al.*, 2024), thus not requiring gene excision. However, transient systems may be very difficult to apply in species that are already highly recalcitrant to transformation. Although wide host range viral systems that can carry out transient editing, such as tomato spotted wilt virus, are known (Liu *et al.*, 2023), to our knowledge, their infectiousness, potential for systemic spread, and rate of editing have not been established for any forest trees.

Wood chemistry and structure

One of the biggest markets for wood is the pulp and paper industry. Wood is largely composed of cellulose, hemicellulose, and lignin, but the lignin fraction needs to be extracted from wood chips in order to obtain high-quality pulp. The extraction of lignin is costly, as it requires the use of harsh chemicals at high temperature. Reducing lignin content or altering its chemical composition in trees would increase pulp yield and, thus, reduce the area of planted trees needed to supply pulp mills. A developing market for wood is in the biorefinery sector, in which lignocellulosic material is enzymatically converted to fermentable sugars to feed the bioeconomy. Also here, lignin is a main hurdle that limits the efficiency of biomass processing – although extracted lignin can also be an energy source or a feedstock for the synthesis of chemicals and materials (Brienza *et al.*, 2024). According to the Food and Agriculture Organization (FAO), the consumption of primary processed wood products is predicted to grow by 37% by 2050 in a business-as-usual scenario and will increase even more in a bioeconomy scenario (FAO, 2022). Clearly, the expected increase in wood consumption will put further pressure on the available space for plantation forestry, justifying rDNA-based efforts to improve the efficiency of wood productivity and processing.

Glasshouse and short-term confined field trials have demonstrated that strong improvements in wood processing efficiency can be achieved using rDNA technologies (reviewed by Chanoca *et al.*, 2019; De Meester *et al.*, 2022b; Li *et al.*, 2024; Zhu & Li, 2024). For example, downregulation of *CSE* in poplar by RNAi reduced lignin content by 25% and increased saccharification by 80–90% in glasshouse-grown trees (Saleme *et al.*, 2017), and 8-month-old, field-grown poplar CRISPR-edited in *CSE* showed a reduced lignin content by 29%, resulting in a 25% increase in saccharification efficiency (Jang *et al.*, 2021). Although these trees could provide obvious environmental benefits, including less chemical use and more pulp per acre of planted forest, the wood products derived from such trees cannot receive an FSC or other forest certification label simply because of the rDNA method used to engineer them, as discussed above (Strauss *et al.*, 2019).

Lignin has many important physiological functions for sustaining upward growth, stem rigidity, and water conduction under tension in the xylem. Thus, strong modification in lignin content may lead to compromised tree health, especially under stressful environmental conditions, limiting the extent to which wood chemistry and physical properties can be productively modified (De Meester *et al.*, 2022b). One means to reduce or eliminate the negative impact of lignin modification is to target lignin modification to specific tissues and cell types. For example, targeted downregulation of lignin biosynthesis in xylem fibers, while keeping vessel lignification intact, largely alleviates the negative effect on tree health while wood processing efficiency is still significantly improved (Gui *et al.*, 2020; De Meester *et al.*, 2021). Using gene-editing, a variety of strong and weak alleles can be created, allowing further 'fine-tuning' of lignin modification. Indeed, while poplar trees with a bi-allelic (full) knockout of the lignin biosynthesis gene *CCR2* could barely survive, CRISPR-based engineering of one knockout *CCR2* allele in combination with a leaky *CCR2* allele resulted in healthy trees with a 25–41% improved wood processing efficiency (De Meester *et al.*, 2020). A recent development is the engineering of lignin types that are entirely new by directing the biosynthesis of alternative lignin monomers in wood cells. These new monomers will be incorporated into the lignin polymer, facilitating biomass deconstruction (Wilkerson *et al.*, 2014; Mottiar *et al.*, 2016; Sibout *et al.*, 2016; Oyarce *et al.*, 2019; Hoengenaert *et al.*, 2022). Such new lignin structures may also improve the valorization of the lignin fraction itself, making lignin easier to fractionate into basic building blocks for the chemical industry (Van den Bosch *et al.*, 2015; de Vries *et al.*, 2021). Multiplex gene-editing or stacking of genes involving wood quality traits (e.g. lignin and wood density) with genes known to improve biomass yield (e.g. for pest resistance (Klocko *et al.*, 2014) or nitrogen metabolism (Jing *et al.*, 2004)), possibly combined with sterility genes to help in the management of gene flow (Strauss *et al.*, 2017), may help to promote a more sustainable wood products industry (Chang *et al.*, 2018; Sulis *et al.*, 2023).

Integration of breeding and rDNA methods

Genetic improvements to adapt trees to new environments or to improve economically important traits depend on the presence of genetic variation in the breeding germplasm (Fig. 2). Forest trees are in the earliest stages of domestication and harbor a great deal of genetic and phenotypic diversity that can be exploited by conventional breeding. For example, in wild *Populus trichocarpa*, lignin content varies from 15.5% to 27.9%, indicating that major gains in wood processing efficiency can be made by conventional breeding alone (Studer *et al.*, 2011). In addition, rare defective alleles for lignin biosynthesis genes (and probably many other traits) are naturally present in the wild germplasm (MacKay *et al.*, 1997; Marroni *et al.*, 2011; Yamamoto *et al.*, 2020), and they can have a large effect on the trait if made homozygous (Vanholme *et al.*, 2013). However, forest tree breeding takes many decades, inbreeding is difficult (dioecious species such as poplar are mostly impossible to self-pollinate), and new sexual crosses to integrate such rare alleles into the genome of elite clones will break up the clone's genetic constitution, thus requiring extra generations of breeding (Vanholme *et al.*, 2013).

In contrast to conventional breeding, rDNA methods such as RNAi or gene-editing are not limited to standing genetic variation; they make it possible to create or insert alleles as homozygous or dominant forms in any elite line without shuffling their genotypes as in conventional breeding. Moreover, they can be introduced in

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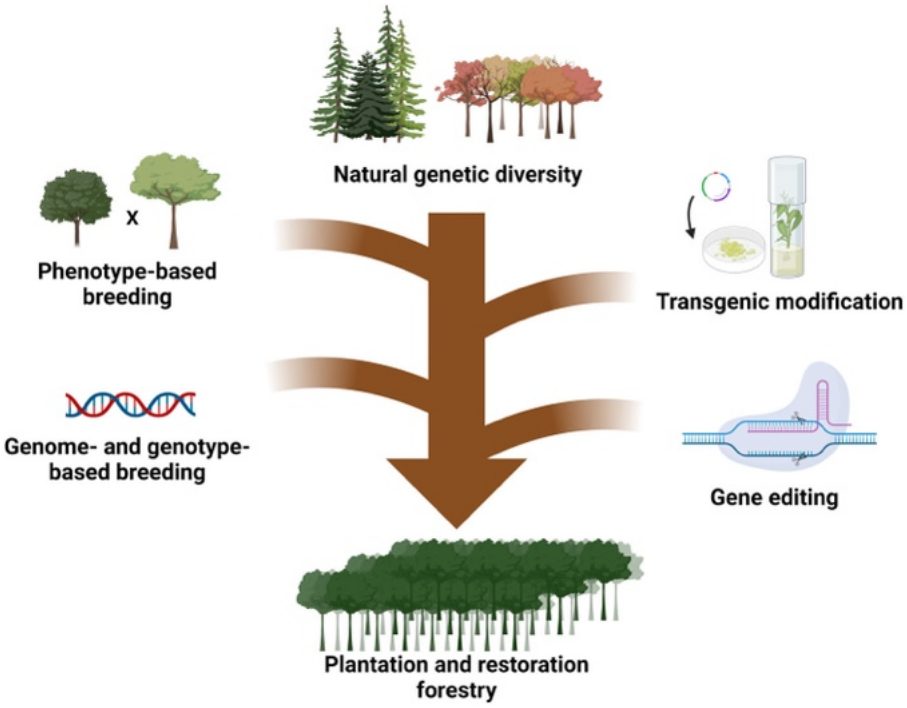

Fig. 2 Overview of methods for genetic improvement of forest trees. To accelerate genetic improvement, conventional and new breeding methods need to be fully integrated, as suggested by the central arrow. Genetic improvement starts by exploiting phenotypic diversity from natural trees and provenances by conventional breeding (sexual crossing followed by phenotypic selection). Current advances in genome sequencing and genotyping allow conventional breeders to accelerate the use of natural genetic diversity using strategies such as genomic selection. A variety of rDNA tools, including transformation (constitutive or tissue-specific overexpression or downregulation using transgenes) and gene-editing (to genetically modify native genes by insertion, deletion, translocation, or inversion), can insert or create desirable alleles for inclusion in breeding populations to modify high-value traits. Figure created with Biorender.com.

multiple parental lines of the breeding germplasm provided that broadly effective transformation technologies are available, as discussed above. rDNA technologies also allow weak alleles to be generated (i.e. not just knockouts or strong overexpression), and these can be readily edited at multiple loci, giving a wide variation of effects that would be sorted out during field studies as part of conventional breeding. Thus, rDNA modification should not be regarded as an alternative to conventional breeding, but rather as a co-developed, complementary tool for generating diversity that is directed at specific traits of interest.

Genetic variation in most traits of economic importance, including yield, crown form, and overall stress tolerance, is controlled by hundreds to thousands of genes. For such traits, conventional breeding, in some cases aided by genomic selection, is the method of choice to improve these highly polygenic traits (Fig. 3). rDNA methods, which require knowledge of specific genes and their roles in the control of valuable traits, are currently best suited for physiologically simpler, mostly qualitative traits such as changes to plant chemistry, sexual fertility, and resistance to selected pests and pathogens that are controlled by a few genes (Strauss *et al.*, 2022). However, successful engineering of ecophysiologically complex traits, such as abiotic stress tolerance or yield, has been demonstrated numerous times in the glasshouse and field, and achieved to a limited extent in commercial agricultural crops such as wheat and maize (Simmons *et al.*, 2021; Gupta, 2024). With the advent of multiplex editing and advanced phenomic methods, the division between traits suitable for rDNA-based modification and conventional breeding may weaken over time.

With the growing demand for wood, and the increasing pressure on forests due to climate change and rapidly changing pest populations, it is especially critical that rDNA technologies are integrated in conventional breeding programs in a manner that does not slow conventional breeding. Most important is the early evaluation of rDNA-modified trees in the field (Fig. 4), preferably at several locations and over several years that approach a harvest cycle, and that the trait is introduced in multiple genotypes of the target commercial species. Currently, most rDNA-based research in forest trees is conducted in laboratories and glasshouses but is rarely further translated through field trials. Contained, indoor studies work well for fundamental research directed toward understanding biological processes and gene function; a well-controlled environment enhances the capacity for the detection of biological effects from a specific genetic modification. However, trees grown in the field experience daily and seasonal differences in weather conditions, including exposure to periods of drought, rain, frost, high UV radiation, wind, and pathogens. Furthermore, they undergo the seasonal cycles of growth and dormancy that have major effects on wood structure and stress tolerance, and also undergo a transition from juvenility to maturity, only starting to flower after several years. This maturation also affects the structure of wood and the expression of most other traits, including tolerance to stresses and pathogens. It is therefore no surprise that the phenotypes of field-grown trees, including their basic chemical composition (Viswanath *et al.*, 2012), are very different from those of glasshouse-grown trees, and that phenotypic differences observed for rDNA-modified trees grown in greenhouse conditions are often not maintained in the field (e.g. Lu *et al.*, 2015; Van Acker *et al.*, 2017; De Meester *et al.*, 2022a, b; Derba-Maceluch *et al.*, 2023). Indeed, conventional breeding, including the statistical model development needed for genomic selection, relies almost exclusively on field results for the same reasons.

Under current regulations and research programs, rDNA-based modifications are usually made in a single model genotype that is

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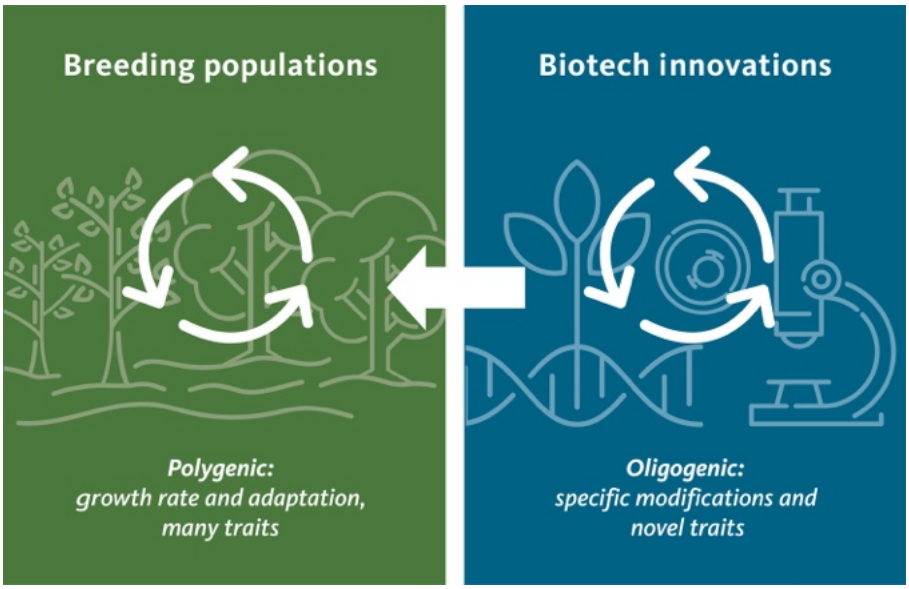

Fig. 3 Integration of rDNA-based modification and conventional breeding. rDNA-based modification is today focused on traits under single gene or oligogenic control and provides qualitative trait modifications (blue box, circular arrows). Conventional and genomic breeding focuses on complex traits that are under polygenic control, resulting in quantitative trait modification (green box, circular arrows). Both forms needs to be efficiently integrated so as not to slow conventional breeding progress. The horizontal arrow represents rDNA trees that enter the breeding program.

easily transformed; thus, rDNA-modified trees are evaluated completely independent of breeding populations. For integration into breeding, the rDNA-based modifications would first need to be recreated in appropriate germplasm and then re-evaluated in field studies in parallel (i.e. as breeding continues to improve germplasm). If the transformation of relevant germplasm and field testing could be readily undertaken as part of initial rDNA concept testing, the real-world value and pleiotropy from rDNA-based modifications could be evaluated much more rapidly (Khaipho-Burch *et al.*, 2023). Successful rDNA-based modifications could then be ready for inclusion in breeding programs soon after, and further tested as part of its crossing and field evaluations.

Early testing is also important because these novel rDNA-based modifications require scrutiny; most have not previously been subjected to multi-generational natural selection as have the highly polymorphic alleles in wild gene pools (Burdon & Walter, 2001).

The few early field studies that have been conducted indeed support their value for rapid evaluation of useful rDNA-based traits. For example, when a variety of RNAi constructs and insertion events were explored in the field with the intent of modifying the xylan content of poplar wood to aid saccharification, several constructs and events were identified that led to favorable results without compromising productivity, whereas others did not result in any improvements (Derba-Maceluch *et al.*, 2023). These

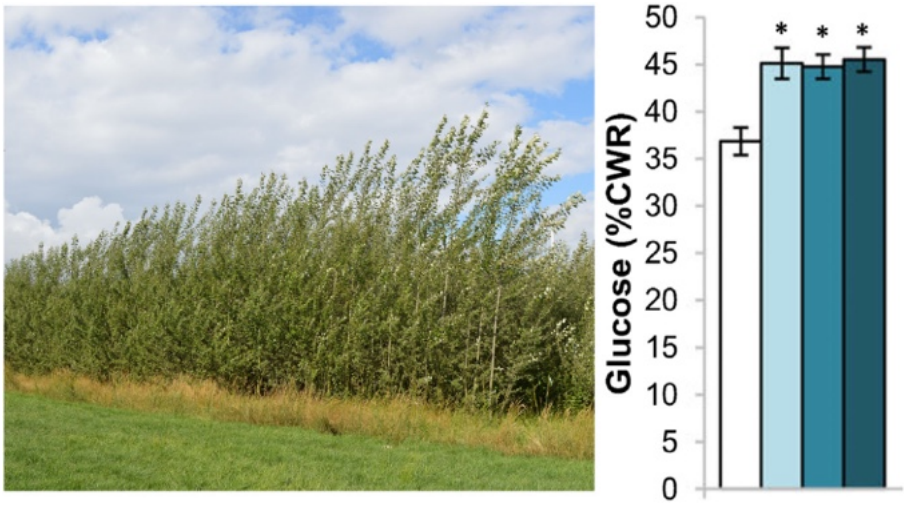

Fig. 4 Field studies of rDNA trees are essential for the integration of rDNA and breeding. Shown is a field trial and saccharification analysis of wild-type (WT) and transgenic *Populus tremula* × *P. alba* with altered lignin composition. Left, trees grown for 2 yr after coppicing. Right, wood from three transgenic lines (blue bars) was found to be easier to deconstruct with cell wall-degrading enzymes as compared to that from WT trees (white bar). Shown is the amount of glucose released as percentage of cell wall residue (CWR). Bars represent means ± SD. Asterisks show that all transgenic lines were significantly different from the WT ($P < 0.05$ for a two-way ANOVA with Dunnett's *post hoc* test). $n = 18$ for WT and all three transgenic lines. Data are from De Meester *et al*. (2022a).

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events could not have been predicted from laboratory studies alone. In a field experiment with poplars intended to identify transgenes that could elevate productivity by modification of active gibberellin levels, only one of several constructs was found to be successful, and would be a logical candidate for further testing and breeding (Lu *et al.*, 2015). Two field trials with transgenic poplar downregulated for *CAD* and with altered lignin demonstrated that the level of *CAD* downregulation is important to balance wood modification with normal growth; modest downregulation resulted in improved wood quality and normal growth, whereas strong downregulation resulted in a strong yield penalty (Pilate *et al.*, 2002; De Meester *et al.*, 2022a). The latter trees had shown no-yield penalty when grown in a well-controlled glasshouse, it was only observed in the field (Van Acker *et al.*, 2017). A similar phenomenon was seen with poplars downregulated for *CCR2* (Leplé *et al.*, 2007; Van Acker *et al.*, 2014).

Putting opportunities and risks in context

Plantation and 'natural forest' biotechnology

Although intensive plantation forestry is likely to be the main focus for rDNA innovations, wild and feral 'natural' trees (including those in forests affected by humans in the past or present) continue to be subject to devastating pathogens and insects for which, in some cases, there is limited innate resistance. This, coupled with the slowness of traditional tree breeding, especially when resistant germplasm is rare, creates a demand for new solutions, potentially including that of rDNA technologies. The most famous case is that of the American chestnut, which was devastated throughout its natural range by an exotic Asian fungal pathogen unintentionally imported to the United States via shipments of plant materials. Despite recent setbacks (Grandoni, 2023) and continuing regulatory obstacles, a transgenic, fully, or partially blight-resistant American chestnut appears likely to be the first rDNA-modified tree used for wild plantings (Newhouse & Powell, 2021). rDNA-based solutions for ecosystem repair beyond the chestnut have been very few, and we know of none that are in advanced field research, which is, to our opinion, due to several factors, including the following: (1) the extensive genetic diversity in forest tree germplasm, which therefore usually includes resistant germplasm, (2) the challenges and costs of transformation technology for most species, (3) the lack of in-depth knowledge of resistance mechanisms and the genes that are needed to support gene-editing or RNAi approaches, and (4) most importantly, the social obstacles in the form of regulatory and market blockages, as discussed above, which severely impede investments in research that could lead to rDNA-based solutions.

Field trials as communication tools

In addition to their essential role in rDNA trait evaluation, field trials are also excellent means for communicating with the public about the science surrounding rDNA-modified trees. Seeing trees in the field – where they generally look like, well, trees!—provides a stark contrast to the caricatured, demonizing picture often painted by activists. Unfortunately, regulations that require strict laboratory and glasshouse containment are the rule throughout most of the world – and make casual viewing of rDNA trees problematic. In some regions, companies are able to establish useful field trials; however, they are not usually open to the public due to proprietary and 'eco'vandalism concerns. Public–private partnerships are likely to be important to enable wider use of field trials for the goals of trusted public education and dialog (May *et al.*, 2024).

Activist concerns about rDNA-modified trees

As forest tree biotechnology causes skepticism among some sectors of the public and the scientific community, it is important to recognize that – similar to tree breeding and other forestry research (White *et al.*, 2007) – assessment of its value and impact will occur incrementally over many years and locations as part of the common forestry practice of 'adaptive management' (e.g. Bolte *et al.*, 2009); the large majority of forest lands and species will see no significant applications of rDNA technologies at all for the foreseeable future. This is a result of the realities of scale-up and replacement of other types of trees and crops as they reach harvest age; the technical and social constraints discussed above; and of the fact that rDNA methods are today mainly suitable for a few intensively grown and clonal plantation species, such as eucalypts and poplars, and a very short list of wild species that are under serious biotic or abiotic threats, such as the American chestnut as discussed above. The realm for gene-editing in forest trees is likewise expected to be very limited for many years, as discussed in depth by Strauss *et al.* (2022). Thus, risks of rDNA use in forest systems will be highly focused, and there will be literally decades for impacts to be assessed and changes in technology or use made if needed. In addition, any risks from rDNA modification will occur along with the growing use and risks from a variety of novel breeding and planting approaches, including assisted migration and species replacement (Gömöry *et al.*, 2020), that are being forced by increasing climate impacts and pest stresses. The risks and benefits of rDNA-modified trees, as well as the risks from their non-use, must be considered in proportion to these very large new risks.

Unlike some activists that regularly emphasize 'genetic contamination' of forests by rDNA trees (e.g. Smolker & Petermann, 2019), we do not consider this, in general, to be a major biological issue. This does not mean that it would not be wise to use genetic containment means (sterility) at scale when socially or ecologically prudent, such as to avoid the spread of herbicide resistance where it would compromise weed control, avoid the spread of an unsafe protein into a food-producing forest tree such as chestnut, or to avoid further spread from exotic forest species that are known to be invasive (Strauss *et al.*, 2017). However, the use of rDNA-based methods *per se* does not, in our view, constitute a sufficient risk to require strict containment, and particularly not at the level of field research and breeding. When rDNA-modified trees could promote environmental values such as stress or disease resistance and thus are potentially critical to sustaining forests, we believe that the risks from some low-level spread into wild or feral trees must be considered acceptable. Indeed, during conventional breeding and ornamental horticulture, genes are continuously

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Box 1 Key requirements for rDNA-based innovations to enhance forest productivity and adaptation

(**1**) Removal of strong regulatory and marketplace impediments to field research and commercial activities;
(**2**) Expanded investment in basic and applied research, including transformation and gene-editing technology;
(**3**) Early integration with breeding programs, including publicly visible and scientifically vetted field studies.

'leaked' from test plantations, generally without significant impact. Of course, until laws and market standards change, the spread of recombinant genes or propagules will present serious legal or market risks and thus severely constrain research.

Conclusions

Forests, both planted and natural, are immense sources of ecological services and sustainable industrial products throughout much of the world. Plantations, the main focus of efforts to employ rDNA technologies, are superior to other forest communities when wood production is the primary goal (Hua *et al.*, 2022), and thus can relieve pressure on native forests for intensive harvesting.

For rDNA biotechnology to help increase forest productivity and adaptation to ongoing rapid climate change, however, it must have a similar freedom to innovate and be deployed to that of conventional breeding. Climate change is well known to existentially threaten forests nearly everywhere, and research in crops suggests that rDNA biotechnology may help in adaptation (Anderson *et al.*, 2020). We are unable to conceive of any of the often-stated risks from biotechnology that are on a par with the risks of doing little to nothing due to the current obstructive social environment. We therefore firmly believe that nothing short of a radical change in regulations, market restrictions, and research investment is warranted (Box 1).

There remain substantial gaps in the technical capacity for efficient use of rDNA technologies in forest trees. This primarily results from the very limited investment from either private or public sources in technology and variety development, including necessary field evaluations, a result that we believe is caused by the daunting social constraints discussed above. Thus, we must have social innovation first if we are to make substantial scientific and technical progress. We therefore should not wait for technology to mature to convince society of the case for strong investments and regulatory relief.

Finally, although acceptance of rDNA technologies in food and forest systems is clearly a 'wicked' problem (Durant & Legge Jr, 2006), we believe that substantial social innovations to regulations and market restrictions are not impossible to achieve. Some of these have in fact occurred in recent years, such as those embodied in the USDA Secure system (Hoffman, 2021) and the new UK Genetic Technology (Precision Breeding) Bill (Caccamo, 2023). Relaxations of regulation are occurring through these new policies, and throughout the world for basic gene-editing, and even the EU is seriously considering revision of its GMO regulatory policies to exempt some categories of gene-edited and cisgenic plants (Nature Plants, 2023). However, we believe that regulatory relief only for simple forms of gene-editing will be woefully insufficient to meet most of the major threats that forests face; a much fuller set of rDNA biotechnologies will need to be included. For example, transgenes or edits that enhance weed management, pest control, pollution reduction, and wood quality have already been demonstrated to provide substantial benefits in pilot field studies (discussed in Strauss *et al.*, 2022). We will need these technologies, closely integrated with conventional breeding in all its forms, to have a chance of significantly mitigating the effects of climate stress on forest health and survival. We also will need to act expeditiously, as the extraordinary pace of climate change in recent years has shown us that time is not on our side. As has been said many times and in many ways (Council for Agricultural Science and Technology, 2013), we believe that today's precaution with regard to rDNA technologies is not to be found in the lengthy delays typically associated with the Precautionary Principle, but in extensive innovation and field research that can provide new options for production and adaptation.

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Competing interests

None declared.

Author contributions

WB and SHS both conceived and wrote the article. WB and SHS contributed equally to this work.

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REVIEW

Reintroduction of at-risk forest tree species using biotechnology depends on regulatory policy, informed by science and with public support

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Abstract
Introduced pests (insects and pathogens) have rapidly increased the numbers of at-risk native forest tree species worldwide. Some keystone species have been functionally extirpated, resulting in severe commercial and ecological losses. When efforts to exclude or mitigate pests have failed, researchers have sometimes applied biotechnology tools to incorporate pest resistance in at-risk species to enable their reintroduction. Often erroneously equated solely with genetic engineering, biotechnology also includes traditional and genome informed breeding—and may provide a holistic approach toward applying genomic-based information and interventions to increase tree species' pest resistance. Traditional tree breeding is responsible for successes to date, but new technologies offer hope to increase the efficiency of such efforts. Remarkable recent progress has been made, and for some at-risk species, novel biotechnological advances put reintroduction within reach. The high costs of reintroduction of at-risk species at necessary scale, however, will initially limit the pursuit to a few species. Successful deployment of pest resistant material may require improved species-specific knowledge and should integrate into and leverage existing reforestation systems, but these operations are sometimes rare where pest threats are greatest. While use of some biotechnologies, such as traditional tree breeding, are commonplace, others such as genetic engineering are controversial and highly regulated, yet may be the only viable means of achieving reintroduction of some at-risk species. Efforts to modify policy toward allowing the use of appropriate biotechnology, especially genetic engineering, have lagged. Provided that risk-benefits are favorable, policy is likely to follow with public opinion; in some countries, society is now increasingly open to using available biotechnologies. Continued engagement using the most recent advances in social science to build public trust, combined with a science-based collaboration among land managers and regulators, will generate the collective momentum needed to motivate policymakers to act rapidly given the speed at which forest health threats unfold and the large areas they affect.

Keywords Biotechnology · Forest restoration · Genetic engineering · Invasive species · Resilience · Tree breeding

Douglass F. Jacobs and R. Kasten Dumroese have contributed equally.

Urgency to reintroduce at-risk forest trees

Introduced pests (insects and pathogens) threaten forests around the world. Due to population expansion that drove associated market globalization during recent decades, the number of introduced (non-native) pests has increased exponentially in many regions (Aukema et al. 2010; Santini et al. 2013). As a result, introduced pests have had a substantial negative effect on forest ecosystems in some regions. In the United States (U.S.), for example, more than 450 alien pest species are present and although most cause minimal damage to forests, about 15 of these pest species account for a tree mortality rate of 5.53 terragrams of carbon (TgC) per year and threaten future loss of 41% of the total live forest biomass (Fei et al. 2019). Similarly, the total economic cost of common ash (*Fraxinus excelsior* L.) dieback in Britain is estimated to be £14.8 billion over 100 years, with more than half of the total cost (£7.6 billion) expected to occur within the next 10 years (Hill et al. 2019). Introduced pests may also negatively impact biodiversity and have important ecological and societal consequences (Mitchell et al. 2018, 2021). In addition to overall forest productivity and biodiversity declines, introduced pests can cause some native tree species to become "at-risk", i.e., threatened by possible exposure to population-level damage, and become of special management concern. In the U.S., tree species are at-risk from about 90 invasive pest species (Alien Forest Pest Explorer 2022). The population level damage for at risk species varies from possible extinction, which is rare, to more commonly innocuous (Williamson and Fitter 1996). However, the 15 or so invasive species that cause severe commercial and ecological loss are vexing (Fig. 1).

Well-known examples of severely threatened keystone at-risk forest tree species include American chestnut (*Castanea dentata* Marshall) Borkh.) due to chestnut blight caused by *Cryphonectria parasitica* (Murr.) Barr (= *Endothia parasitica* (Murr.) Anderson & Anderson); butternut (*Juglans cinerea* L.), due to butternut canker disease (BCD) caused by the fungus *Ophiognomonia clavigignenti-juglandacearum* (*Ocj*;



Fig. 1 At-risk native forest tree species of North America showing pest decline, including (from left to right): white ash (*Fraxinus americana* L.) showing larval feeding galleries under bark from the emerald ash borer, American chestnut (*Castanea dentata*) infected with chestnut blight, and stem canker on butternut (*Juglans cinerea*). Photos: DF Jacobs

Nair, Kostichka, & Kuntz); common ash (*Fraxinus excelsior* L.) in Europe due to ash dieback from *Hymenoscyphus fraxineus* (Kowalski) Baral, Queloz & Hosoya (=*H. pesudoalbidus*; basionym *Chalara fraxinea*); ash (*Fraxinus* spp.) in North America due to emerald ash borer, *Agrilus planipennis* Fairmaire; and elms (*Ulmus* spp.) due to the introduction into Europe and North America of the pathogen *Ophiostoma* spp. that causes Dutch elm disease. The list of new, at-risk species continues to expand rapidly (Potter et al. 2019); for instance, whitebark pine (*Pinus albicaulis* Engelm.), a keystone species in the western U.S. was recently listed as threatened, in part because of the introduced pathogen *Cronartium ribicola* A. Dietr. (U.S. Fish and Wildlife Service 2022).

Often these at-risk tree species remain extant (e.g., individually surviving through stump sprouting or regeneration via seedlings or saplings not yet old or large enough to be affected) but are no longer sufficiently abundant and/or of adequate size and stature to reproduce sexually or perform their ecological function(s), rendering them ecologically extinct (McCauley et al. 2015, 2017). Ecological extinction, the sufficient loss of a tree species' ability to influence ecological dynamics (Tilman 2001), is insidious, as obligate species in higher trophic levels become extinct first, causing deleterious, cascade effects to the ecological web (Wardle et al. 2000; Säterberg et al. 2013; Mitchell et al. 2014). These altered ecosystems may experience a lower diversity of plant dispersal and tree recruitment (Redford 1992) that leads to a homogenization of forest ecosystems, which has negative implications for forest resilience to perturbations, including changes in climate. Left unchecked, these at-risk tree species may develop truncated ranges, lose genetic diversity, and face becoming threatened, endangered, or extinct (Potter et al. 2017, 2019).

The well-known historical loss of some foundational tree species has led to novel predictive and prioritization capacity (Potter et al. 2019) for addressing pest threats to at-risk species. Theoretically, the most effective approach should be exclusion or reducing the rate of spread of pests, but this has not been well adopted due to both international and intra-national constraints. For example, despite the early detection of emerald ash borer threats to *Fraxinus* spp. in North America, campaigns and regulations to eliminate inter-state transfer of logs or wood and sanitation removal of infected trees were unsuccessful in stopping the spread of the pest, which is now ubiquitous across much of North America where ash occurs and has decimated ash tree populations (McCullough 2020). With few remaining alternatives to exclude threats to at-risk species of high commercial and/or ecological value, researchers worldwide have implemented disease resistance programs to develop and deploy pest resistant trees back to the landscape (Sniezko and Koch 2017; Woodcock et al. 2018, 2019).

Some of the longest running such programs began between the 1950s–1980s to develop pest resistance in species such as American chestnut, Sitka spruce (*Picea sitchensis* (Bong.) Carrière), American elm, and white pines (i.e., *Pinus monticola* Douglas ex D. Don, *P. strobus* L.) and have shown some success (Sniezko and Koch 2017; Woodcock et al. 2019). Other disease resistance programs, such as for ash in response to emerald ash borer, have developed very recently. A medium to long-term approach and sustained investment are necessary to have potential for operational deployment of resistant trees using traditional tree breeding (Woodcock et al. 2019; Sniezko and Nelson 2022). Recent advances in tree biotechnology, however, have potential to accelerate the pace and breadth of pest resistance to support reintroduction of at-risk species.

Advancements in biotechnology put reintroduction in reach

Biotechnology refers broadly to the use of biology to solve problems (Editors of Encyclopedia Britannica 2022). In the context of at-risk forest tree species, biotechnology to reintroduce species can take many forms (traditional and genome informed breeding and genetic engineering or the synonymous term, genetic modification). Biotechnology is often erroneously equated solely with genetic engineering. Distinguishing among these forms may help to elucidate the multi-faceted nature of biotechnology for incorporating pest resistance and leverage all available biotechnologies to support reintroduction. This approach may provide a holistic framework toward applying genomic-based interventions to increase tree species' pest resistance efficiently and effectively (Sniezko and Liu 2022; Nelson 2023). The need and use of these biotechnologies will depend on context associated with specific at-risk species (Dumroese et al. 2015). Informed and judicious use of biotechnologies can provide an avenue for rescuing at-risk, ecologically extinct species and moving them toward their restoration on the landscape. In addition to the aforementioned facets of biotechnology, other biotechnologies not discussed herein, such as biological control, may be integrated into pest resistance programs.

Traditional tree breeding is a biotechnology that may leverage other biotechnologies, such as use of rooted cuttings, grafting, and somatic embryogenesis to duplicate trees with desired characteristics (Merkle et al. 2023; Nelson 2023; Fig. 2). In traditional tree breeding, genotypes of the at-risk species or of closely related species with desired traits are conserved in vitro or in situ (Engels et al. 2008) and bred for multiple generations using controlled pollination and subsequent selection of resulting progeny having favored characteristics. This leads to combinations of genes that could occur "naturally" toward obtaining the desired resistance. On the one hand, traditional tree breeding has limitations because trees (especially conifers) have extremely large genomes, are slow to reproduce, and often multiple genes and their interactions with the environment are responsible for the desired trait(s) leading to a low heritability and slow accumulation of genetic gain over time (De La Torre et al. 2014). A single breeding cycle often requires a decade or more to complete (Harfouche et al. 2012). Additionally, due to its non-targeted nature, breeding for a desired trait (e.g., disease resistance) may result in accumulation of undesirable traits as well. For instance, growth of American chestnut seedlings bred with Chinese chestnut for blight resistance has not matched that of pure American chestnut in field plantings, despite several generations of backcrossing with American chestnut (Brown et al. 2022). This is likely because backcross hybrid trees selected for blight resistance inherent on average 17% of their genome from Chinese chestnut (Westbrook et al. 2020). On the other hand, traditional tree breeding remains a powerful tool with broad social acceptability to rapidly enable development of resistance, especially for tree species with extensive diversity of resistance mechanisms and high cultural value (Luiz et al. 2023), and recent successes are encouraging (Sniezko and Koch 2017).

Recent and rapid advances in *genomics*, for example, DNA sequencing, genetic marking with molecular markers, and bioinformatics are providing geneticists with novel, powerful, potentially time-saving tools that have potential to assist to reintroduce at-risk species. Genome sequencing of trees provides a basis for better understanding resistance (i.e., frequency, level, distribution, type, durability, stability) toward describing genes with putative disease resistance (e.g., Harper et al. 2016; Stevens et al. 2016; Stocks et al. 2019). Analyzing the RNA transcripts produced by the genome (transcriptomes) has potential to inform levels and mechanisms of resistance within a species as well as species within the

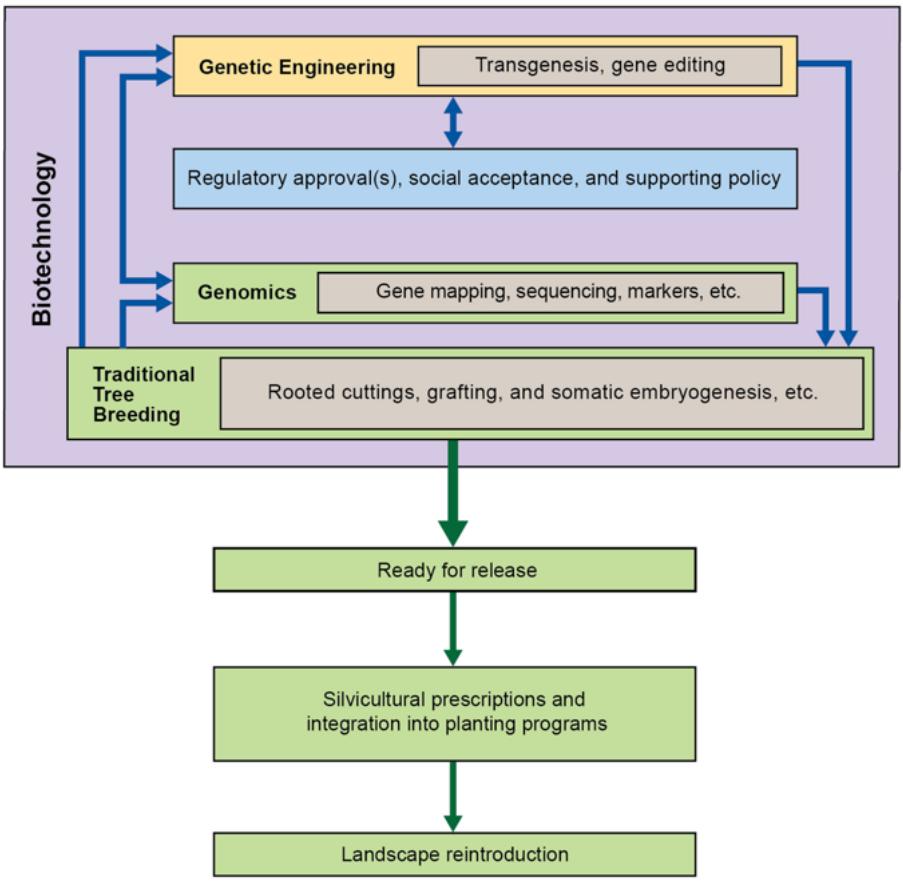

Fig. 2 Biotechnology may take many forms, with varying regulatory frameworks, and provides a holistic approach toward applying genomic-based interventions to increase tree species' pest resistance. Deployment of pest resistant material achieved through biotechnology to support landscape-scale reintroduction is dependent on capacity to integrate into operational tree planting programs, silvical knowledge, societal acceptance, and public policy

genus (Sniezko and Liu 2022). Genetic mapping and marker-assisted selection can assist in identifying genes conferring major resistance, complexes of genes that together provide elevated quantitative resistance, and describe genes that contribute to increased pest susceptibility (Engelhardt et al. 2018; Sniezko and Liu 2022, 2023). This information can be leveraged toward stacking multiple genes into a single genotype (i.e., pyramiding) to improve the durability of resistance (Sniezko and Liu 2022, 2023). Moreover, all these techniques can be used to further evaluate characteristics of the pest, rendering information on the mechanisms used, and potential adaptation of those mechanisms, by the pest (e.g., Rigsby et al. 2015; Duan et al. 2017). These biotechnological techniques offer promise for more rapid and robust selection of genotypes for inclusion into traditional tree breeding programs. Achieving the full potential of these techniques will depend on overcoming social, biological, and economic constraints (Whetten et al. 2023).

As discussed previously, *genetic engineering* is often erroneously equated with biotechnology yet is in fact simply a set of specific tools within biotechnology. We consider

genetic engineering as "any technique that uses recombinant, synthesized, or amplified nucleic acids to modify a genome" (Nelson 2023) and any plant derived with such modified nucleic acids, be they cisgenes or transgenes, to be a genetically engineered organism. Notably, however, this field is moving rapidly in response to regulatory and policy changes. In the U.S., an emphasis on mechanisms of action rather than gene insertion methods or specific insertions is increasingly determining whether or not the result of genetic engineering could have been achieved through traditional tree breeding (Federal Register 2020), which affects the regulatory process. The revolutionary use of CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats) gene editing technology, which can accurately and relatively easily insert and alter DNA with targeted specificity, has intriguing implications for the pyramiding of resistant genes as well as removal of genes associated with elevated levels of susceptibility (Gorash et al. 2021). While application of CRISPR was recently achieved in European chestnut (*Castanea sativa* Mill.) (Pavese et al. 2021), technical and societal obstacles to its near-term use remain. These obstacles include a lack of knowledge to guide gene editing for traits of complex inheritance, difficulty of transformation in many important genotypes, and complexity of application in breeding programs (Strauss et al. 2022). Additionally, field trials are highly regulated in most countries and recombinant DNA-modified trees are not allowed in key forestry certification programs (Strauss et al. 2022).

Leveraging or refining silvicultural systems to accept pest resistant trees

To successfully deploy pest-resistant germplasm to reintroduce at-risk forest tree species, planting of nursery-grown seedlings will be needed to artificially regenerate these species. An understanding of the biology and ecology of these species is needed to ensure successful artificial regeneration that may lead to re-establishment of pest resistant populations with potential to naturally regenerate across the landscape (Jacobs 2007; Jacobs et al. 2013). This species-specific foundational information can be drawn upon to guide seed collection, nursery seedling propagation, site preparation and planting techniques, as well as to manage regeneration after its reintroduction into forests. The existing knowledge base varies among species, often depending on the length of time that a given species has been extirpated from the landscape. For instance, American chestnut and American and European elms were functionally eliminated from forests decades before the development of modern principles of forest ecology (Paillet 2002) and only recently has field-based research been directed toward informing restoration efforts (Jacobs et al. 2013; Wang et al. 2013; Martín et al. 2019). Other species, such as ash in North America or Europe (Pautasso et al. 2013; McCullough 2020), have more recently been threatened by pests and therefore may already have well-established methods, including planting requirements, to draw from. For some other recently threatened forest tree species, however, regeneration has mainly been accomplished naturally and prescriptions for planting establishment of such at-risk species may still be poorly developed, yet this knowledge is needed to successfully deploy pest-resistant material.

In addition to establishing a sound knowledge base to facilitate reintroduction of at-risk forest tree species, deployment of pest-resistant germplasm through artificial regeneration could logically be accomplished by integrating into and leveraging existing systems of tree planting operations. However, > 90% of forest regeneration in the U.S. is accomplished

naturally (Oswalt et al. 2014) and at-risk species in need of reintroduction may be lacking the capacity for operational nursery production and planting in respective regions of high priority for deployment. In the case of the continental U.S., for example, the highest occurrences of pests (i.e., threats) are sometimes located in regions with the lowest rates of tree seedling production in forestry nurseries as well as total land area planted (Fig. 3). This trend is most notable in the northeastern U.S., which as a global hub for trade imports has historically high risk of pest introduction (Fei et al. 2019), yet its forest management relies nearly entirely on natural regeneration for stand establishment (Bataineh et al. 2013). The southeastern U.S. has by far the highest rates of seedling production and planting

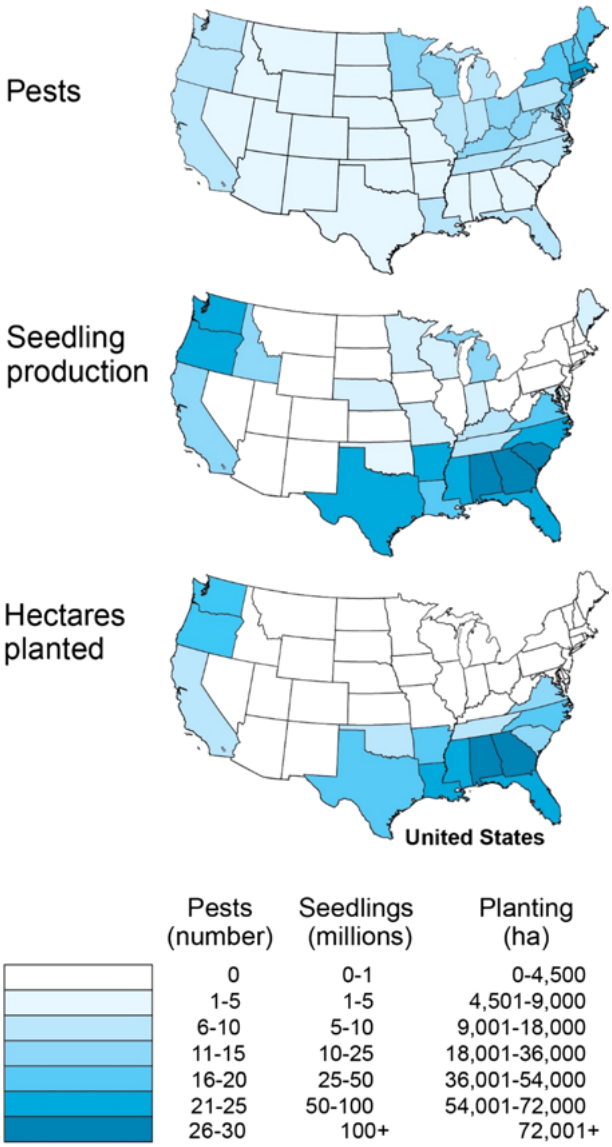

Fig. 3 The cumulative number of pests by state putting forest trees at-risk in the coterminous United States (Alien Forest Pest Explorer 2022), annual forest tree seedling production, and subsequent land area planted (Haase et al. 2021)

associated almost exclusively with commercial pine plantations (Fox et al. 2007), but relatively lower forest pest occurrences. Although reforestation systems in the southeastern U.S. rarely target at-risk forest tree species, the existing nursery and planting infrastructure could more easily accommodate such a shift than in the northeastern U.S. This presents a dilemma to solve to ensure that pest resistant material can be successfully deployed across the landscape.

Societal perception and policy remain weakest links

Moving forward with policy that supports using biotechnology (ranging from selective breeding to genetic engineering) to reintroduce at-risk tree species will be challenging. The ensuing discussions and ultimate decisions will need to consider, meld, and communicate across a spectrum of science disciplines, a disparate public with a continuum of personal values, knowledge, and perceptions toward the topic, regulators having important responsibilities to the public, and policymakers keen to satisfy constituents. Traditional, longstanding, and widely used tree breeding to produce disease resistant trees for reintroduction is characterized by general acceptance among the public and relatively few policy regulations in their use. Nevertheless, social acceptability of deploying specific products derived from traditional tree breeding, such as use of hybrids or backcross trees, is not entirely resolved as some uncertainty and reluctance among land managers and the public remains (Brennan et al. 2023; Jacobs et al. 2013). Using genetic engineering to reintroduce at-risk tree species is much less well accepted, although regional and demographic variation exists (Brennan et al. 2023; Jepson and Arakelyan 2017a, b; Marzano et al. 2019). For example, younger generations appear to be more open to use of genetically engineered trees in natural woodlands and forestry plantations (Jepson and Arakelyan 2017a, b; St-Laurent et al. 2018). Here, we focus the discussion on the challenges and opportunities of framing the use of all forms of biotechnology to reintroduce at-risk tree species.

The public perception of and associated policy regulations of using genetic engineering in plants is primarily focused on food crops and is complex and contentious (Hallman et al. 2003; Costa-Font et al. 2008; Frewer et al. 2013; Jepson and Arakelyan 2017a; Marzano et al. 2019). On one hand, the public may more readily support the use of genetically engineered trees because trees are valued for reasons beyond food, such as for watershed protection, wildlife habitat, carbon sequestration, and conservation (Merkle et al. 2007; Gamborg and Sandøe 2010; Brister and Newhouse 2020). On the other hand, the public may be more hesitant to accept genetically engineered trees because trees are longer-lived and therefore have longer-term ecological implications (Williams 2005; Hall 2007; Merkle et al. 2007; Gamborg and Sandøe 2010). Indeed, public acceptance of using genetic engineering to reintroduce at-risk tree species may be lacking because the intention is to have heritable results that persist across generations. This contrasts with other biotechnologies, such as with human medications, that limit effects to an individual and enjoy widespread public favor (Aucott and Parker 2021).

Whether the topic is climate change, microplastics, reclaimed water, genetically modified organisms, or just about anything else, humans often form opinions based on little scientific data and a range of perceptions (e.g., Wunderlich and Gatto 2015; Ricart and Rico 2019; Catarino et al. 2021). These opinions are constructed on values that lie on a continuum from egoistic (concern for self), to altruistic (concern for community), to biospheric (concern for non-human species) (Stern 2000). Not surprisingly, environmental values also lie on a continuum, with support for pro-environmental policies generally thought to

increase with movement from egoism to biospherism (Vaske and Donelly 1999). This simplistic approach becomes murky, however, because people perceive aspects of the environment, such as nature, naturalness, wildness, and health, through a localized prism that also includes community values and culture (Hull et al. 2010). Thus, for example, an egocentric individual could support a pro-environment policy perceived to provide local benefit to the person or community, whereas a biocentric individual, who believes that forests have an intrinsic value regardless of value to humans, may oppose a pro-environment policy that is perceived to challenge the notion that "nature knows best" and can "self-heal" (Hull et al. 2010). This distinction can be more fluid because an individual could, depending on circumstances, express all three values and be pro-environment (Snelgar 2006). Such value orientations, once expressed, can be slow to change (Hiroyasu et al. 2019).

Many popular opinions are made based on little scientific knowledge, and increasingly influenced and reinforced through social media. However, public knowledge concerning a particular topic or policy also runs a continuum from vague or general awareness to more specific, detailed knowledge (Trevethan 2017). Similar knowledge continuums are also evident among professionals within disciplines. For example, within the U.S. Department of Agriculture (USDA), Forest Service, land managers often have a different working knowledge (and perceptions) concerning climate change amongst themselves (Rodriguez-Franco and Haan 2015) and compared to agency scientists actively researching the topic. And, within the science community engaged in exploring genetically modified organisms, other continuums exist. For example, research molecular biologists may approach non-knowledge (defined as the absence of knowledge) from a control-oriented perspective (unknowns can be explored in the controlled atmosphere of the laboratory) whereas research ecologists do so from an uncertainty-oriented perspective because unknowns are often characterized by complex systems with high spatial and temporal heterogeneity that span diverse disciplines (Böschen et al. 2006).

As noted above, acceptance and support of forest management activities are influenced by an individual's values; often the public focus on historical or static forest conditions precludes acceptance of novel or unconventional approaches perceived to be unnatural and therefore riskier or unethical (Park and Talbot 2012; St-Laurent et al. 2018). Value-based decisions are tempered, however, with the amount of knowledge one has and the level of trust one has in that information (McFarlane et al. 2006; Hajjar and Kozak 2015; Gregory et al. 2016). Public opinion must be respectfully considered and integrated because adoption and implementation of any forest management activity depends on public trust (McFarlane et al. 2012; Greenberg 2014), and levels of trust fluctuate with levels of knowledge and experience. As an individual increases their knowledge and experience with a topic, the basis of decision-making shifts from trust in perceived experts who can make informed decisions to a greater reliance on personal judgement (McFarlane et al. 2012).

Developing trust in the message is vital, and scientists and environmental groups are perceived with the highest public trust on other contentious forestry topics, such as assisted migration, with the federal government, industry representatives, and politicians perceived less so (St-Laurent et al. 2018; Solano et al. 2022). Trust in scientists may translate to less perceived risk and more acceptance of interventions, but the delivery of data may or may not be helpful. Schuler (2004) concludes that for food GMOs, public perceptions, and not science, were paramount in framing the discussion, and although information can shift perceptions, it is not easy, nor the only factor (Satterfield et al. 2009).

Understanding that communication is foundational to success is much simpler, and much different, than actual, effective communication with the public that garners, for example, support for using biotechnology to reintroduce at-risk species. Given the complex

nature of the debate, and the wide spectrums of values and knowledge across a disparate group of actors, how might scientists converse with others toward obtaining social acceptance to safely, ethically, appropriately, and holistically use tools in the evolving biotechnology toolbox?

Listening to concerns and engaging the public are paramount for building trust. In western Canada, impacts from insects and diseases were perceived to be most detrimental to the well-being of forest-dependent communities (Hajjar and Kozak 2015; St-Laurent et al. 2018). Building on this, thoughtful dialogue with the public about how introduced insects and diseases put native tree species at risk, and how biotechnology may be part of a holistic approach to reintroduce those species, should begin immediately and be ongoing (Clark et al. 2002; Powell et al. 2019). Members of the public are more prone to agree with others who share their values; thus, to be successful the messaging must be delivered from a broad spectrum of advocates, thereby positively influencing their disparate factions. A major challenge is finding the correct spokesperson(s) because who delivers science-based information is critical (Kahan 2010). Moreover, the message from the spokesperson must be true and truthfully (without hidden bias) presented (Andersson et al. 2006). The likelihood of a single message coalescing from diverse advocates is, however, unlikely (Sylvester et al. 2009). Even so, the responsibility likely falls to government to forge a coalition toward development of the public process to transparently, and in balanced fashion, discuss critical issues to assess the use of biotechnology, and thereby avoid, or begin to address, non-science-based opinion (Cobb and Macoubrie 2004).

While the public trusts federal government less than scientists (St-Laurent et al. 2018), a federally supported initiative that leads with scientists and leverages its partners and stakeholders around a central theme that addresses and provides for diverse values may be an effective strategy. For example, the USDA Forest Service, which has a long tradition of managing forests to meet multiple use objectives, could lead communications in the United States. The agency has a cadre of scientists and university partners focused on molecular biology, genetics, ecology, and restoration, as well as NGO partners, who together could provide information (pros and cons) that the public trusts. The agency currently supports environmental, industrial, NGO, and other partners toward successfully obtaining mutually agreed upon diverse management objectives, such as biodiversity, rural community development and sustainability, recreation, clean water provision, and healthy, resilient forests. Thus, a message from scientists and a trusted locally based spokesperson could speak to how all of the various facets of biotechnology may (or may not) serve as a tool to reintroduce populations of at-risk trees.

In creating dialogue concerning at-risk trees, a focus on ecological and societal benefits rather than risk management may be prudent (Hiroyasu et al. 2019), as is providing opportunities for the public to participate in decisions concerning research and development (Burri and Bellucci 2008; Barnhill-Dilling et al. 2021). It will also be essential to operationalize on-going communication across governmental agencies that ultimately share responsibility for regulating and implementing the use of biotechnology. While a land management organization may promote the ecological and social benefits more than risk management per se, regulatory organizations, with their fiduciary responsibilities to society, namely a "do no harm" approach to implementation of genetic engineering, must necessarily focus on risks (Martín et al. 2019; Pierce et al. 2023); a regulatory process that balances safety, development, and use could prevent unintended ecological consequences without severely limiting use (Gordon et al. 2021). These two approaches need not be antagonistic; realistic discussion associated with ecological processes along with perceived benefits must be supported with data and provide a clear pathway for resolving bias based

on popular opinion. Acknowledging that tree breeding programs are complex and that researchers must consider unintended consequences are important for building public trust (Martín et al. 2023). The use of forecasting (e.g., simulation models that evaluate potential outcomes of reintroduction prior to deployment) may afford a better discourse about potential trade-offs associated with using biotechnology, especially across scales and including opportunity costs (including ecosystem services) associated with or without implementation (Mozelewski and Scheller 2021). Recent work confirms that public and cultural attitudes about perceived benefits and risks are strong drivers of acceptance of trees genetically engineered for resistance to introduced pests (Barnhill-Dilling et al. 2020; Petit et al. 2021; Brennan et al. 2023).

The message shared, especially concerning genetic engineering, must be presented with concrete rather than vague or abstract language; eliminating jargon (e.g., Plaxco 2010) and providing specific examples (Tallapragada et al. 2021) improves delivery effectiveness. For instance, when St-Laurent et al. (2018) surveyed western Canadians about the generic, undefined use of genetically engineered trees in reforestation, the result was poor support for the practice. When more nuanced, specific, described applications of biotechnology in reforestation in the central U.S. were communicated, including genetic engineering, it garnered high levels of support among land managers (Brennan et al. 2023). Provision of scientific knowledge delivered by a trusted locally based spokesperson allows individuals to make more nuanced decisions (Hiroyasu et al. 2019), which may be important for the topic of rescuing at-risk trees using genetic engineering where emphasizing ecological benefits may be paramount to acceptance, and for developing necessary grassroots support that influences policy makers (Daniels et al. 2012). Research is clear that engaging the public and increasing awareness have generated support for other contentious forest management practices, such as increasing the use of prescribed fire (e.g., Loomis et al. 2001) and assisted migration (St-Laurent et al. 2018). It is also clear that public awareness and perceptions are critical to land manager willingness and ability to implement management practices (Archie et al. 2012).

The literature shows that the information deficit model of simply educating the public (Nisbet and Scheufele 2009) is no longer the sole solution to garnering support for use of biotechnology when and where appropriate. While scientist engagement with the public is paramount and can ensure a scientifically ethical approach, equally important are science delivery and deliberate, collaborative dialogue among land managers, regulators, and policy makers (Barnhill-Dilling et al. 2021; Harfouche et al. 2021). Natural scientists likely need to re-evaluate their approach(es) to public engagement, recognizing that it is not just a lack of information, but rather their lack of commitment to be enablers of broader, direct public engagement and dialogue that is counterproductive to the needed effort (e.g., Besley and Nisbet 2011).

There is a need to identify and implement ways to inclusively weave diverse stakeholders into the decision-making process (especially at the local level; Kofler et al. 2018), from research priorities to citizen science opportunities to policy advocation (e.g., NASEM 2017, 2019; Barnhill-Dilling et al. 2021). The solution should no longer be based on the pretense that scientists should "give the public more information so that they can rationally agree with us," but rather to foster conversations that include and respect diverse perspectives, creating broad support that can result in long-term support for complex tree breeding programs for at-risk species. During the last decade, the biology and science related to biotechnology approaches in tree breeding has made remarkable progress. Efforts to affect policy toward allowing the use of biotechnology have, however, lagged. While investing resources into the biological side of biotechnology is essential, a concomitant investment

by natural scientists into the social science side of biotechnology may be even more important. Support across the full spectrum of the public (i.e., scientists, land managers, stakeholders, regulators) will hopefully motivate policymakers to react (Daniels et al. 2012).

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Declarations

Conflict of interest The authors declare no conflicts of interest.

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