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

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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

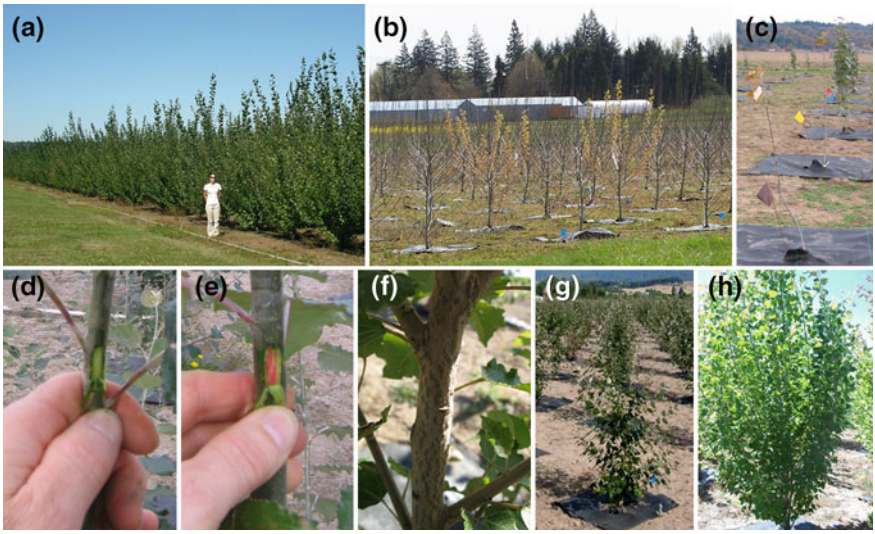

Fig. 2 Gene tagging. **a** Program Manager Liz Etherington with field trial of activation-tagged trees in 2009. A variety of phenotypic alterations were observed in these field trials, including, **b** early bud flush, **c** early leaf senescence, **d**–**f** altered wood color and bark texture (**d**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

◀ **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

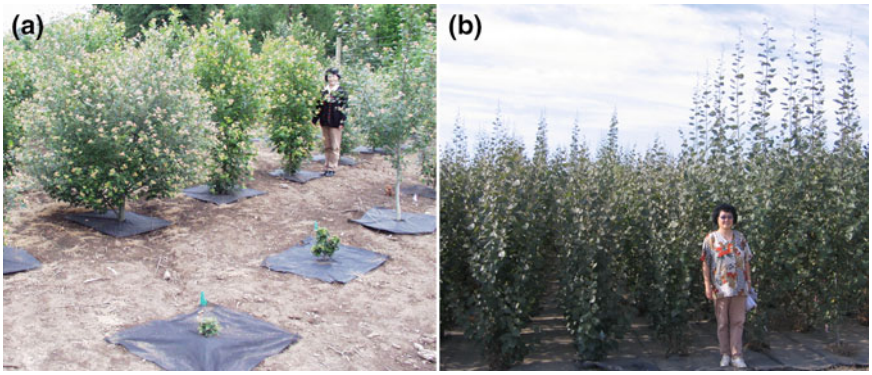

Fig. 4 Form and architecture modification. Dramatic alterations in **a** tree shape (bushy vs. narrow, *back row*) and size (dwarf, *front row*) and **b** stature of GA-modified poplars

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

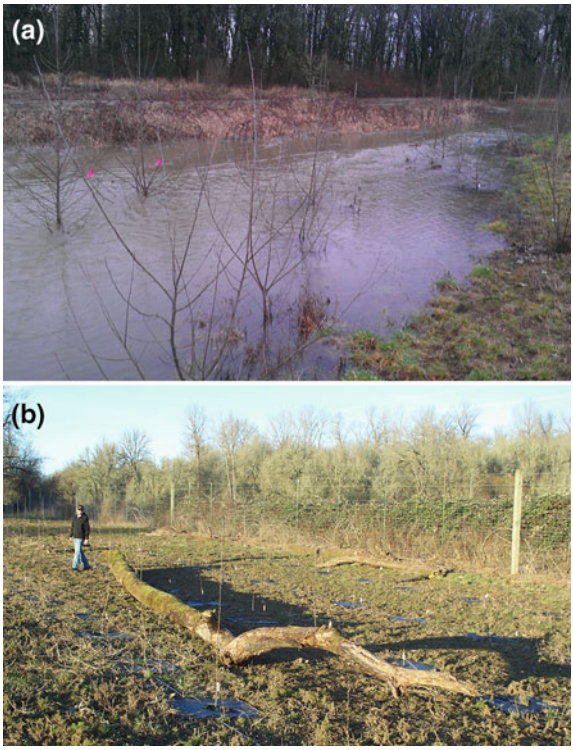

Fig. 6 Field site disturbances. **a** Flooding of a field site by a nearby river, **b** large debris brought in by flood waters. All trees were monitored, accounted for, and the event promptly reported to the USDA

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,

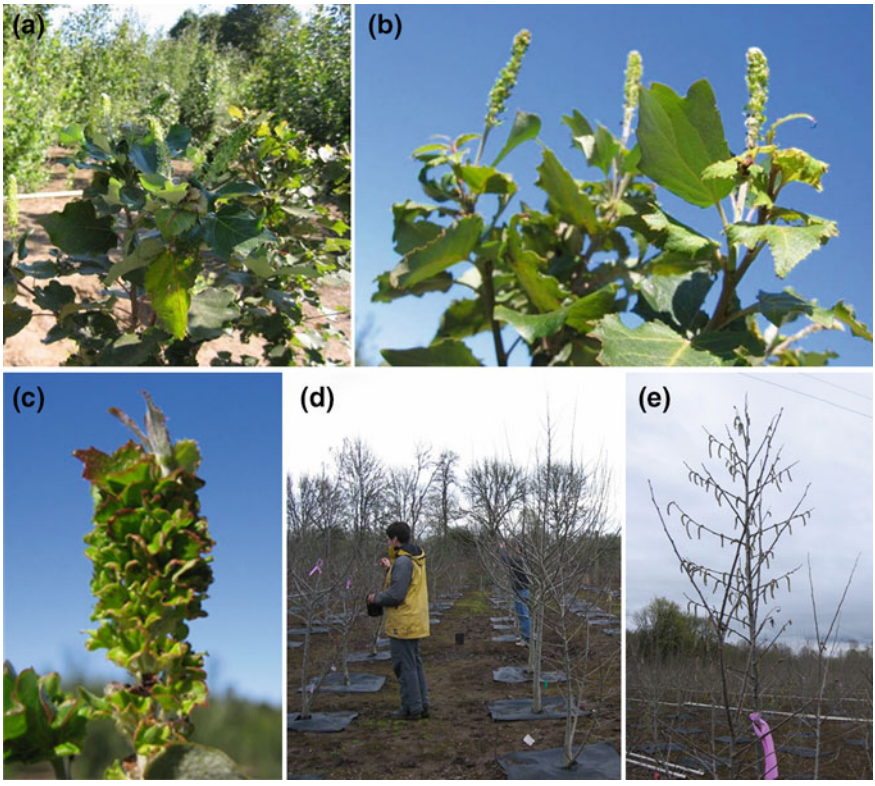

Fig. 7 Collection of unexpected flowers. Trees in one trial flowered unexpectedly in the summer, and our permit requirements (which did not allow for flowering) necessitated removal of every single flower. **a** Trees with fully expanded leaves began to develop flowers. **b** Unusually, flowers grew 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.

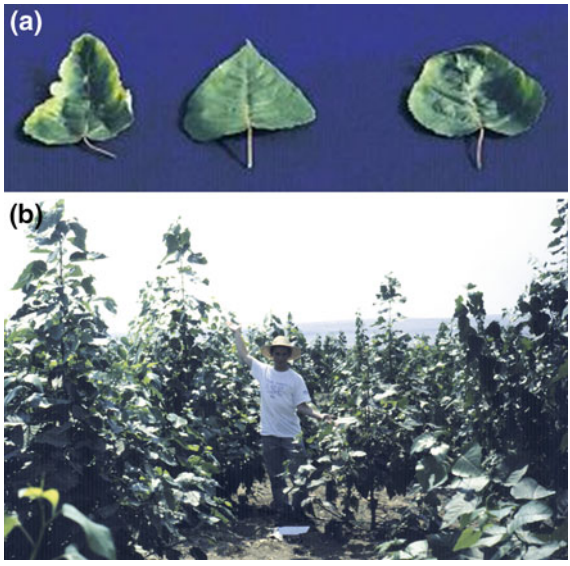

Fig. 8 Rare tree abnormalities observed in transformation events with hybrid cottonwoods. **a** Leaves with variable shapes and mottling (Ault et al. 2016: control in *center*), and **b** a dwarfed mutant (*right*) compared to majority of transgenic trees (*left* and rest of plantation)

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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