DEVELOPMENT & VALIDATION OF STERILITY SYSTEMS IN TREES

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Plan for today

- Context / why sterility systems
  - Challenges
- Examples of evolving science
- Methods for engineering sterility
- Examples of progress under 2020 project
  - DNM sterility systems
  - RNAi sterility systems
  - Field trials

Tree biotechnology for renewable materials and bioenergy

REVIEW

The Path Forward for Biofuels and Biomaterials

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Biomass represents an abundant carbon-neutral renewable resource for the production of bioenergy and biomaterials, and its enhanced use would address several societal needs. Advances in genetics, biotechnology, process chemistry, and engineering are leading to a new manufacturing concept for converting renewable biomass to valuable fuels and products, generally referred to as the biorefinery. The integration of agroenergy crops and biorefinery manufacturing technologies offers the potential for the development of sustainable biopower and biomaterials that will lead to a new manufacturing paradigm.

We are apt to forget the gasoline shortages of the 1970s or the fuel price panic after Hurricane Katrina, but these are but harbingers of the inevitable excess of growing demand over dwindling supplies of geological reserves. Before we freeze in the d

future reductions in the ecological footprint of energy generation will reside in a multifaceted approach that includes nuclear, solar, hydrogen, wind, and fossil fuels (from which carbon is sequestered) and biofuels. These concerns have also been advanced by the

make a major contribution?" One answer, coming from a forum at the 27th Symposium on Biotechnology for Fuels and Chemicals, was that some applications are ready now, but their impact will be limited with current technologies and feedstocks (*8*). We need commercialization and policy support for current and near-term opportunities to grow the industry from its present base. Equally important, we need research and development to increase the impact, efficiency, and sustainability of biorefinery facilities. The current production and use of bioethanol and biodiesel processes are a starting point. It is our belief that the next generational change in the use of bioresources will come from a total integration of innovative plant resources, synthesis of biomaterials, and generation of biofuels and biopower (Fig. 1).

Innovative Plant Design via Accelerated Domestication

"More, Bigger, and Better," the mantra of modern consumerism, also summarizes—ironically—the goals of research aimed at modifying plant species for use in sustainable biomass production. Interrelated plant traits ... ture, resilience

Biorefinery concept

Multiple products from biomass via engineering of feedstock and its processing

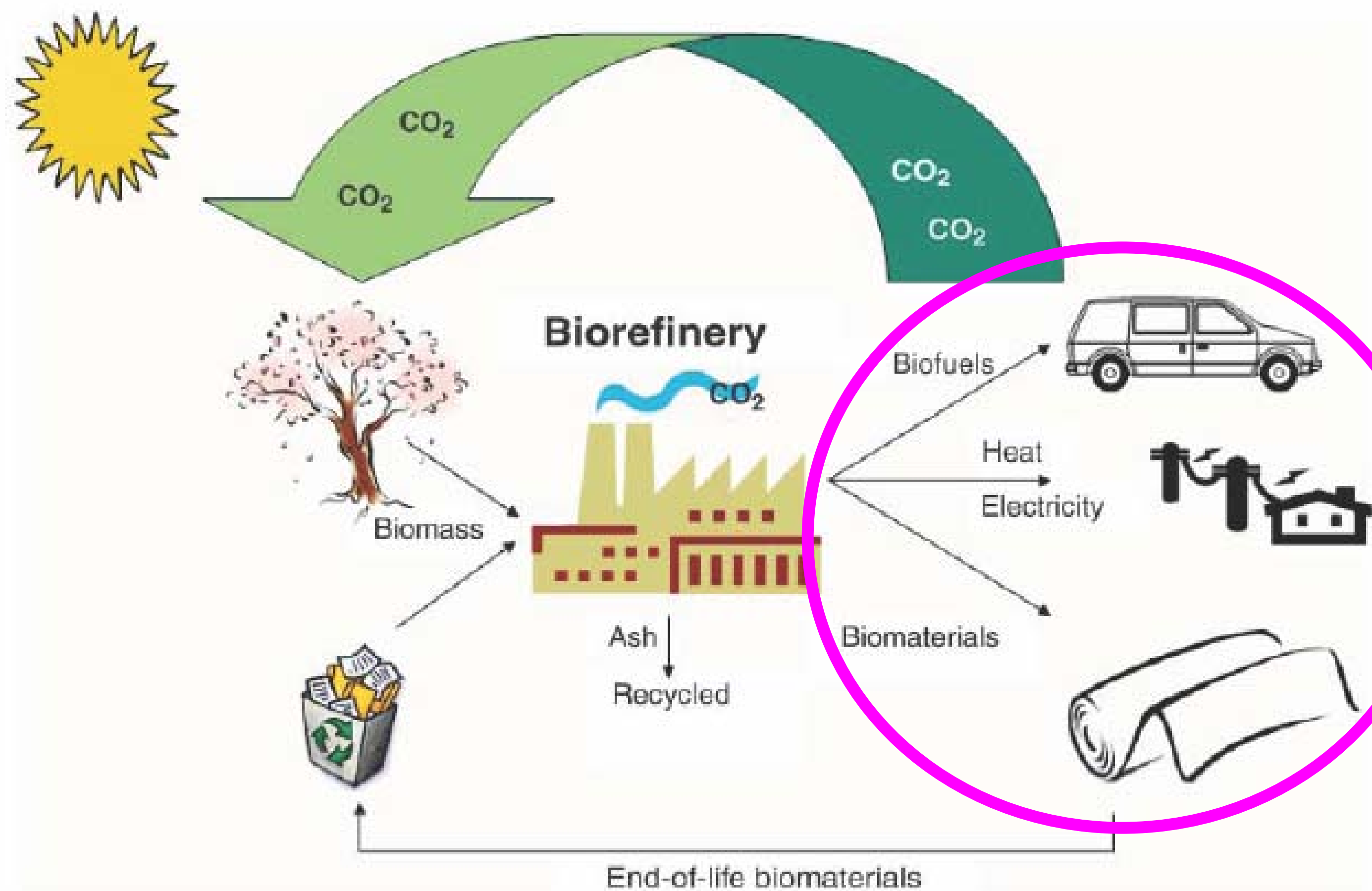


Fig. 1. The fully integrated agro-biofuel-biomaterial-biopower cycle for sustainable technologies.

Ragauskas et al., Science 2006

Sterility a domestication goal

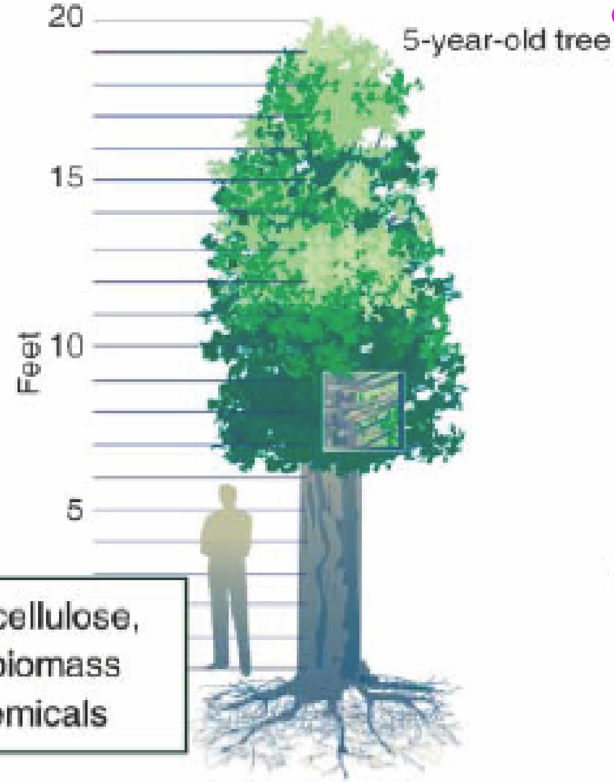


Fig. 2. Overview of plant traits that can be targeted by accelerated domestication for enhanced plant biomass production and processing.

Ragauskas et al., Science 2006

System of interest: Poplars as models and for wood farms

Poplar an extraordinary model tree for biotechnology & ecophysiology

- Full genome sequence, large EST sequence databanks
- Facile transformation (gene insertion) into selected genotypes (aspens and their hybrids)
- Facile clonal propagation to replicate genotypes
- Rapid growth

Best place to test concepts and technologies in any tree

Why sterility?

- Strongly reduce risks of exotic organisms and genes
  - Long distance dispersal
  - Ecological complexity
  - Long time frame for “invasion” to occur
  - Trees as ecologically dominant species
  - Dispersal entropy high: ~Irreversibility
  - Evolutionary changes, co-evolution
- Effects and spread technically unpredictable
- Essential for public acceptance of biorefinery, multiple product genotypes?

An enabling technology for all genetic engineering in bioenergy crop systems?

Why containment?

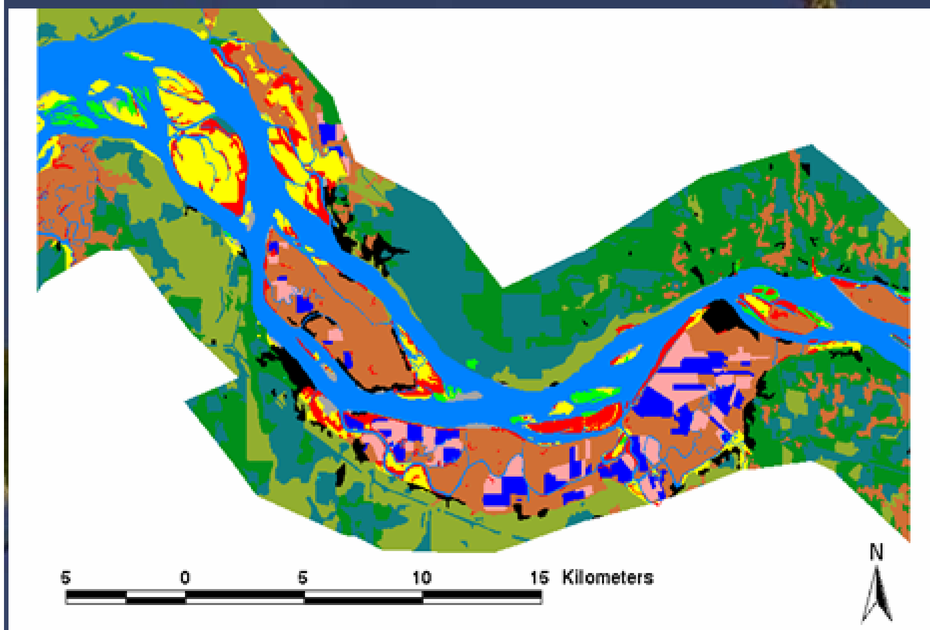
- Promote coexistence of different social values
  - FSC forest certification system, and organic agriculture system, bans GE trees and crops
- Stewardship
  - If it can be done with reasonable cost, delay, ecological impact, and is socially acceptable, should we not do it?
  - Feasible: Vegetative propagation of completely sterile genotypes facilitates engineering– compared to complex “terminator” systems under study ag crops with seed/fruit product

Is containment possible?

- Will require years of field trials and monitoring
  - Key motivation for this project *now*
- Pursuing various methods in parallel as its unclear what is best
  - Rapid evolution of science and technology gives new options, dictated changes in approaches
- Part of risk assessment, but critical for risk reduction to low levels

Key role in risk assessments

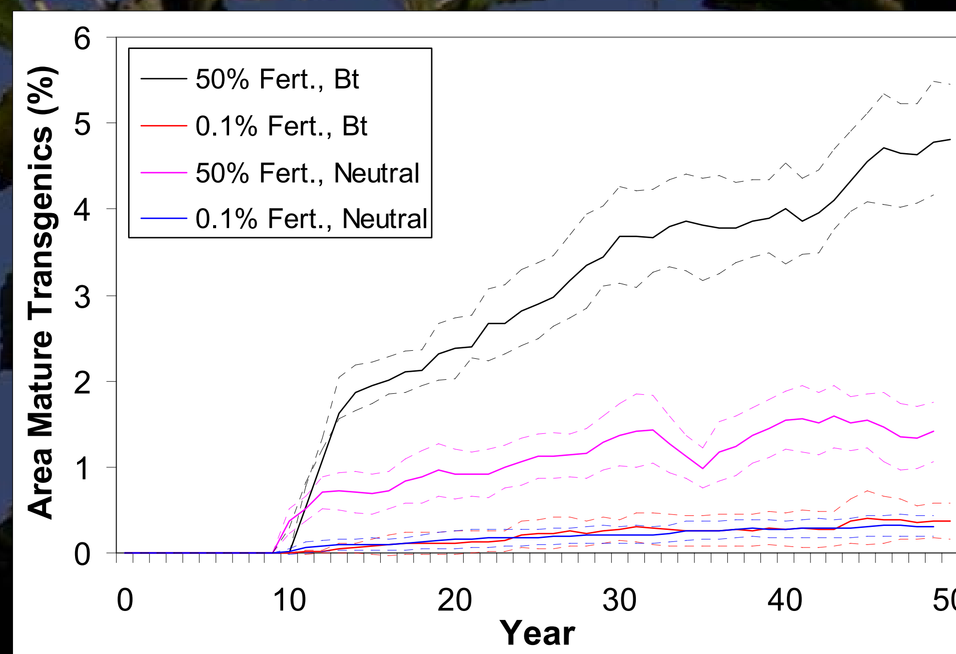
Even modest sterility can greatly reduce gene flow – poplar case study in Pacific Northwest



Columbia River, Northwest USA

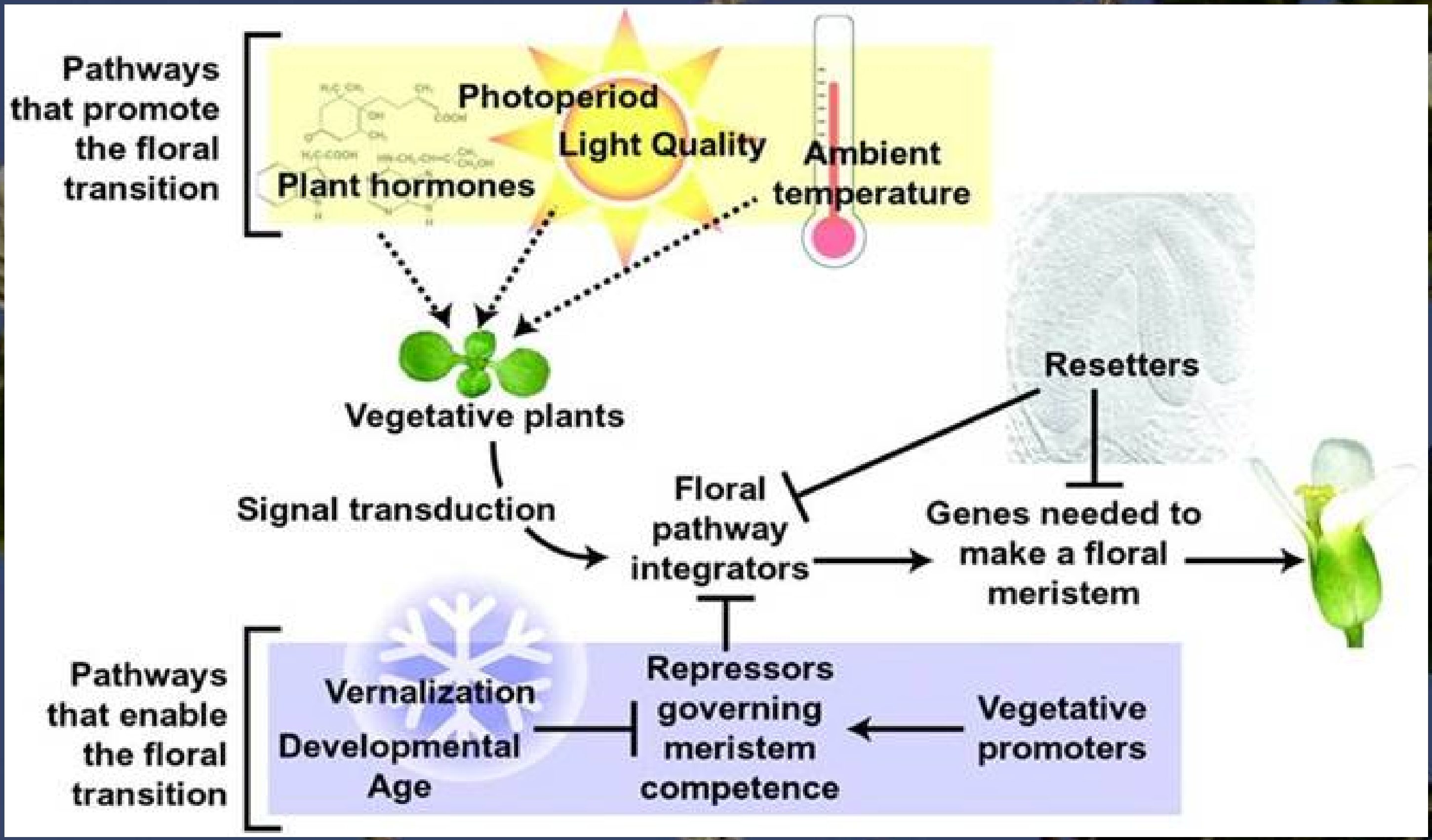
DiFazio et al. 2004

Favorable transgene prevalence, 50 yr



Rapid science & technology development

Arabidopsis, comparative genomics of flowering, the foundations



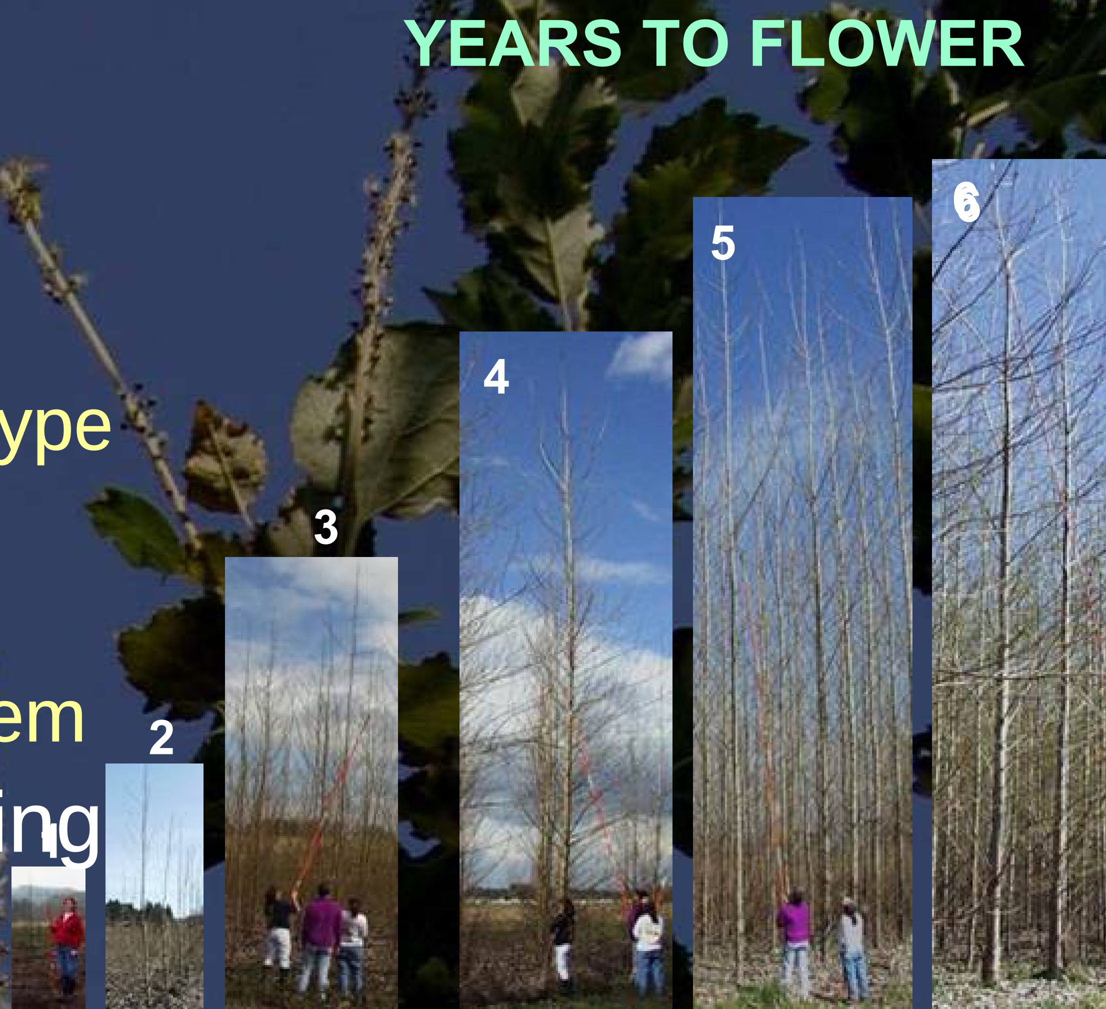
Many pathways, many new options, continually being identified

Phylogenetic & ecological diversity

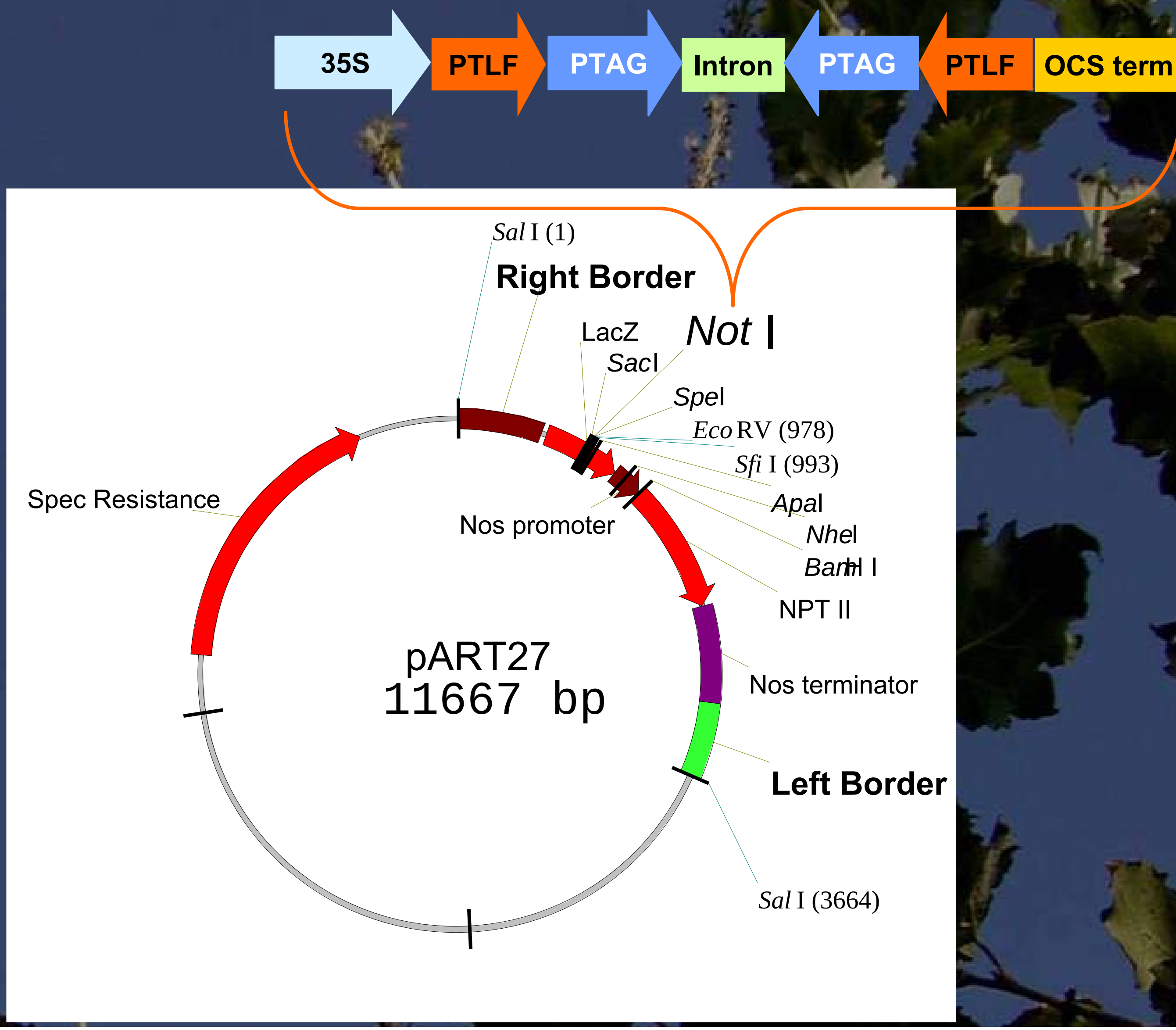
Henderson IR, Dean C. 2004. Development 131: 3829-3838.

Significant technical barriers to progress

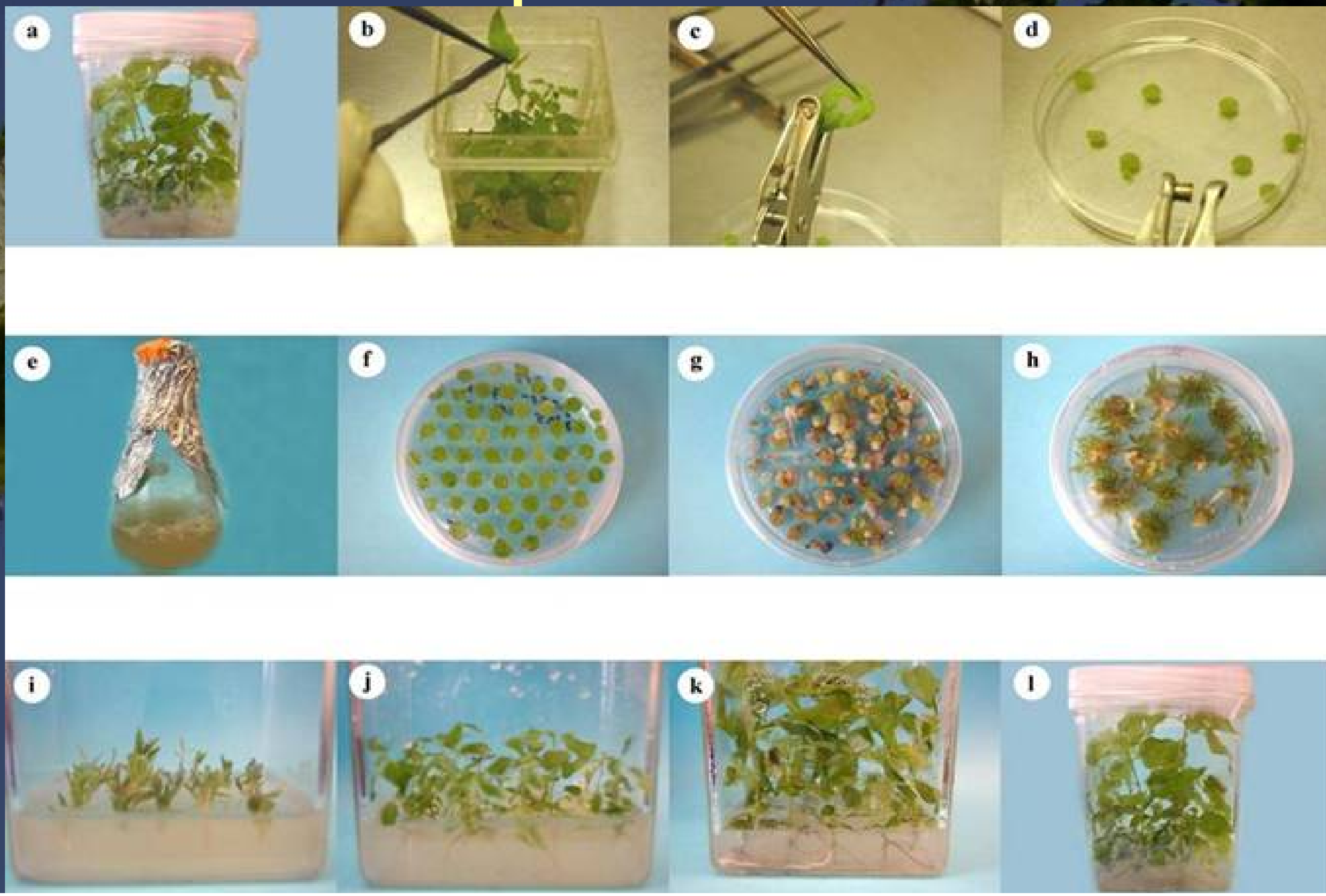
- Gene constructs complex
- Large scale transformation, propagation
  - Male/female/genotype
- Flowering delay !
  - Early-flowering, transformable system
- Tree size at flowering
- Costs of field studies
- Changing regulations



Sterility constructs often complex



Poplar transformation & propagation requires ~12-18 months until ready to plant in field



Many attempts to create model early-flowering tree system

35S-LFY / Poplar *AGAMOUS* co-transformation phenotype

Catkin on 8-month-old *Populus alba* 6K10

Field flower induction

Catkins on 9-month-old willow

Flowering 10-month old *Eucalyptus occidentalis* and transgenic plants

Transgenic tobacco / Arabidopsis

Science progress and associated research approaches – "3 generations"

First generation sterility transgenics

Genes that encode tissue-specific floral proteins derived from other species

Pollenless trees: Tobacco tapetum promoter *TA29 fused to Barnase*

2nd generation

Bisexual, poplar derived floral homeotic and meristem identity genes

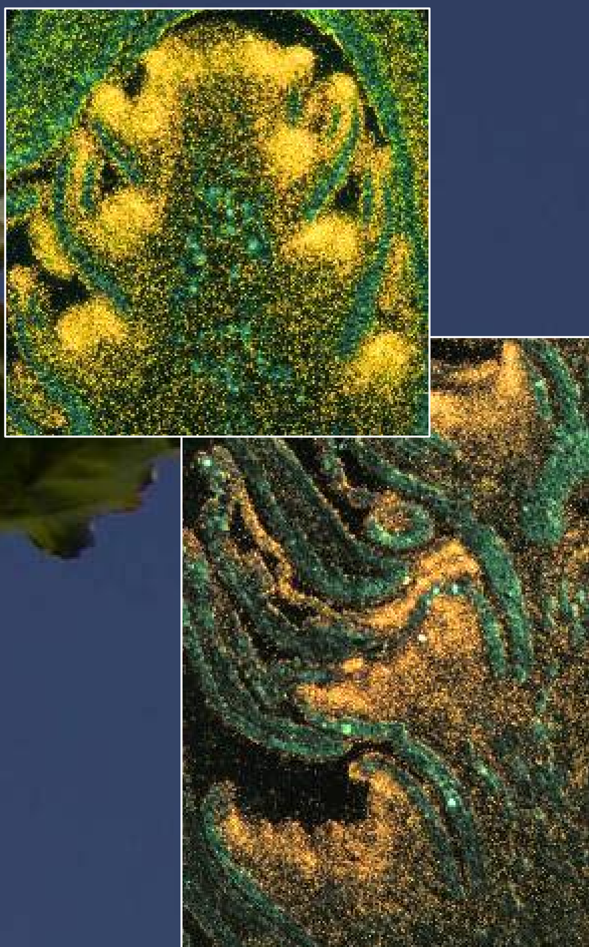
Arabidopsis Gene	Function in Arabidopsis	Poplar Homolog(s)
***** ***AGAMOUS (AG)***	**Stamen & carpel identity**	***PTAG1*** ***PTAG2***
***** ***APETALA3 (AP3)***	**Petal & stamen identity**	***PTD***
***** ***APETALA1 (AP1)***	**Flower initiation;** **perianth identity**	***PTAP1-1*** ***PTAP1-2***
LEAFY (LFY)	**Flower initiation**	***PTLF***

***MADS-box gene, member of a large plant gene family**

In situ hybridization to verify expression pattern of poplar homologs

Expression in male catkins

PTLF



PTAP1-1

PTAG

3rd generation: Poplar transgenes for <u>preventing</u> transition to flowering

(EST, poplar genome sequence)

Arabidopsis Gene	**Function in Arabidopsis**	**Poplar Transgene***
TFL1	Represses flowering	***35S::PCENL1***
FT	Promotes floral transition	***PFT-RNAi***
SVP	Represses flowering	***35S::PSVPL***
AGL24	Promotes floral transition	***PAGL24-RNAi***
AGL20	Promotes floral transition	***PAGL20-RNAi***
FPF1	Promotes floral transition	***PFPF1-RNAi***

*** Co-transformation of multiple transgenes for redundant systems**

Delayed flowering constructs entering field trials – poplar homologs

Based on EST/genomic sequence

- RNAi
  - *PAGL24*
  - *PAGL20*
  - *PFT*
  - *PFPF1 (2)*
  - *PFT/PAGL20*
  - *PFT/PAGL20+PFPF1*
  - *PMFT*
- Overexpression
  - *PCEN*
  - *PSVP*
  - *PAGL24*


Technological approaches to
engineering containment

Methods for engineering containment

1. Mitigation genes (reduced fitness)
   - Reduced stature (GA, gibberellin inhibition)
2. Ablation
   - Floral promoter::cytotoxin fusions
3. Suppression of essential genes
   - Directed mutation / deletion
   - Transcriptional / post-transcriptional suppression (RNAi)
   - Protein interference (dominant negatives)
   - Structural or floral onset gene candidates
4. Overexpression of floral repressor genes to keep plants in vegetative phase

Redundancy within/between mechanisms

Reduction of tree height also a goal for domesticated bioenergy trees

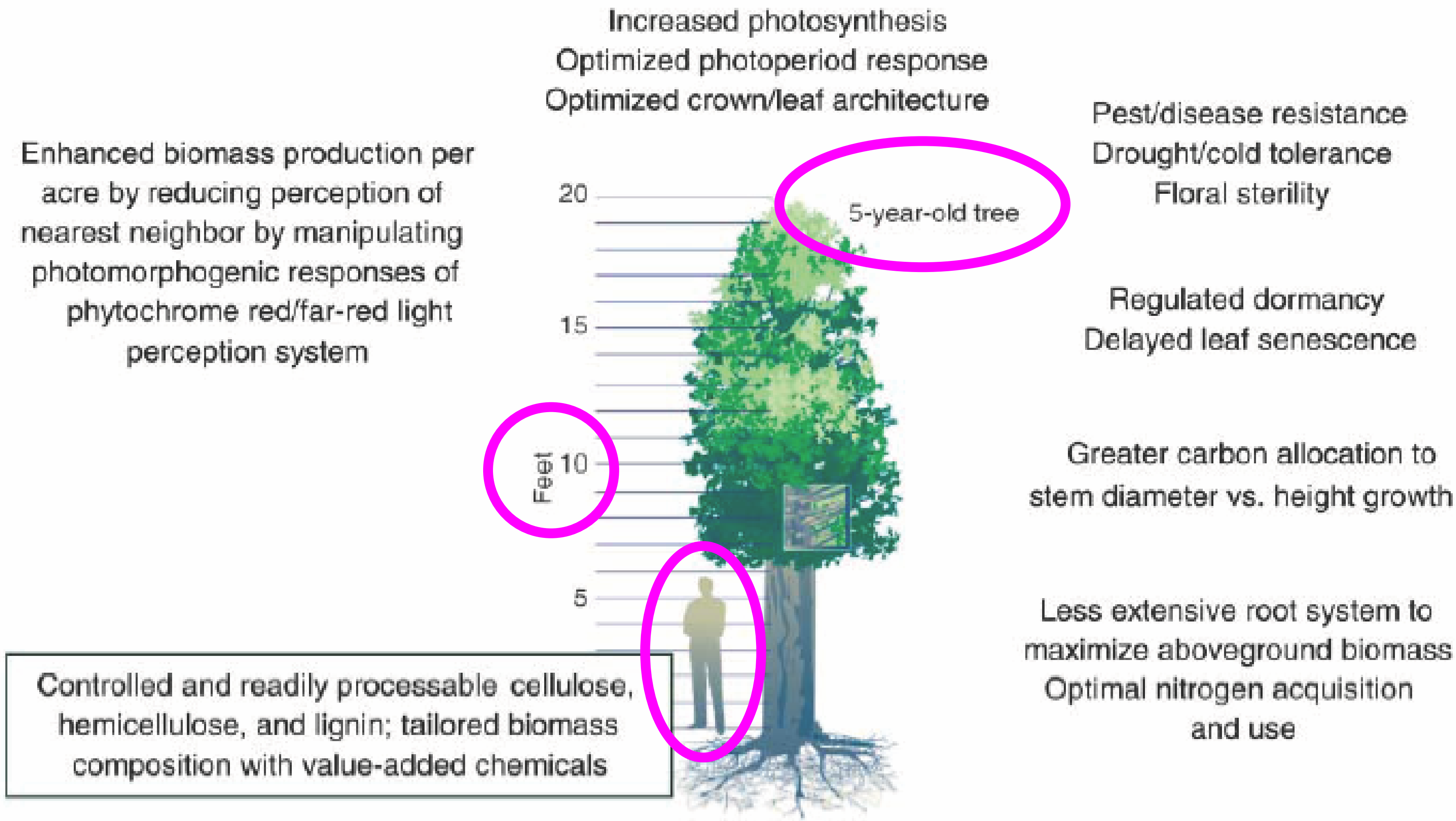
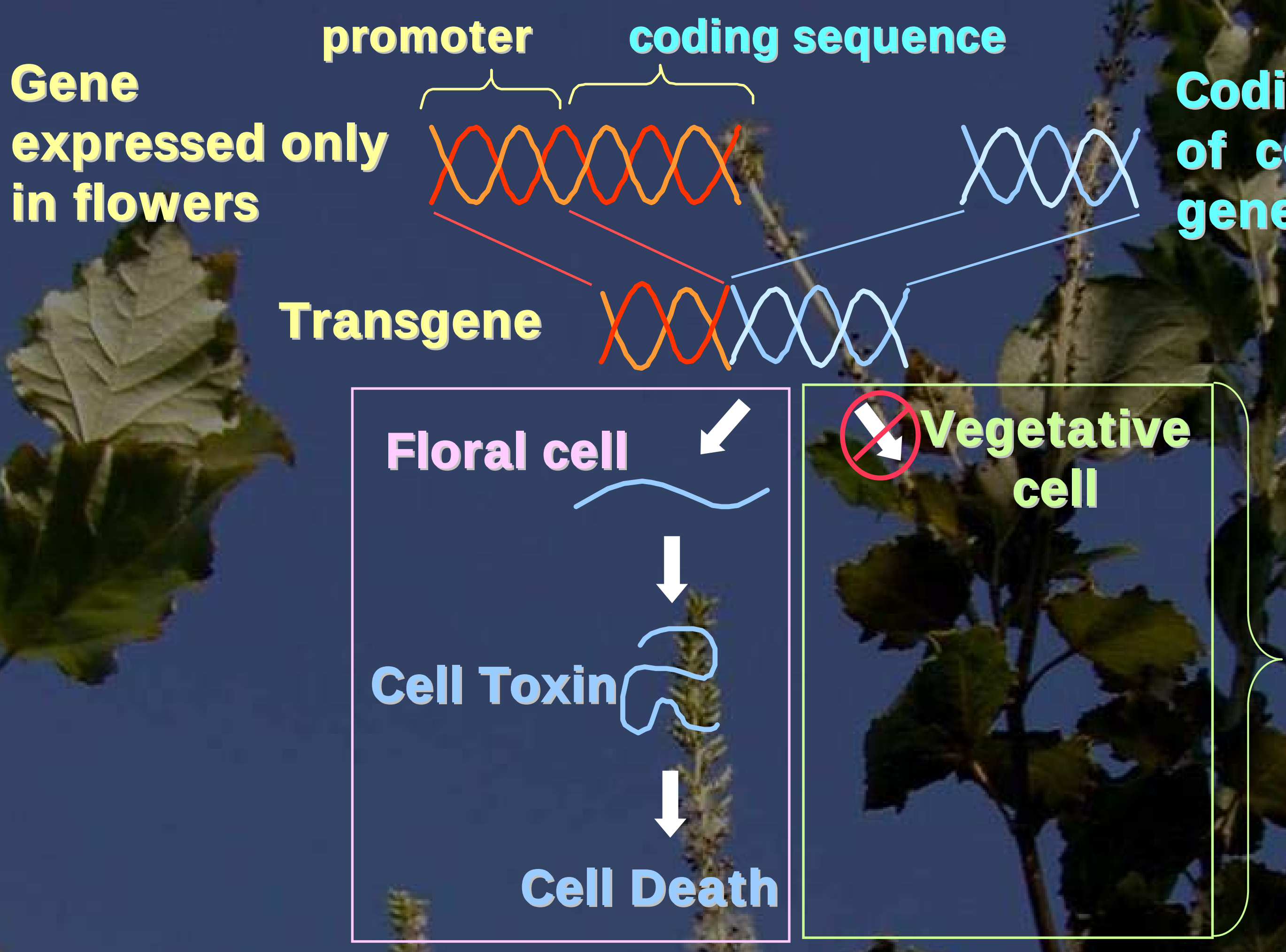


Fig. 2. Overview of plant traits that can be targeted by accelerated domestication for enhanced plant biomass production and processing.

Rasguskas et al., Science 2006

Mitigation: GA modification genes as tools for modifying tree form

Floral ablation (cell death)

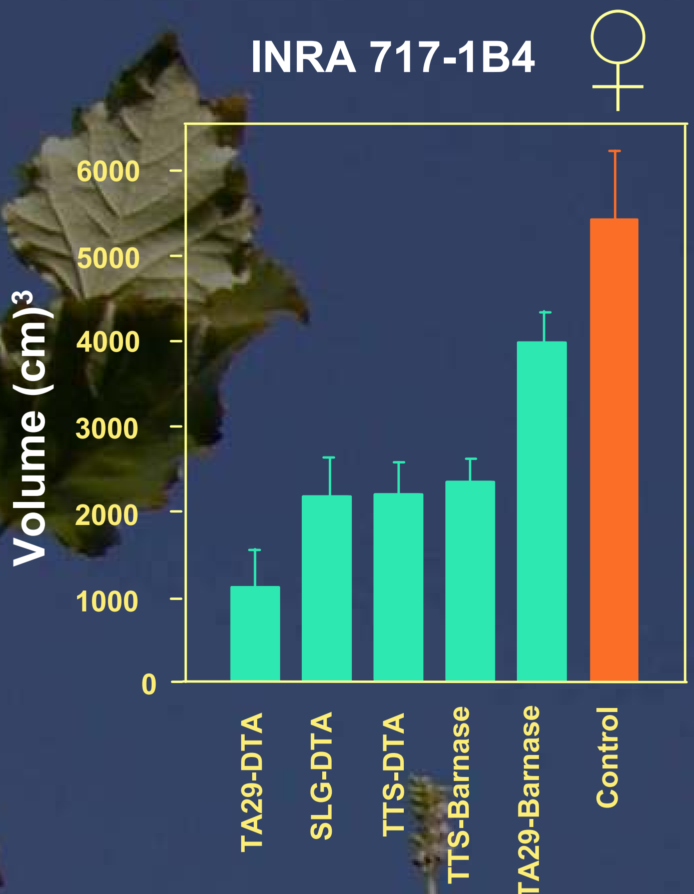


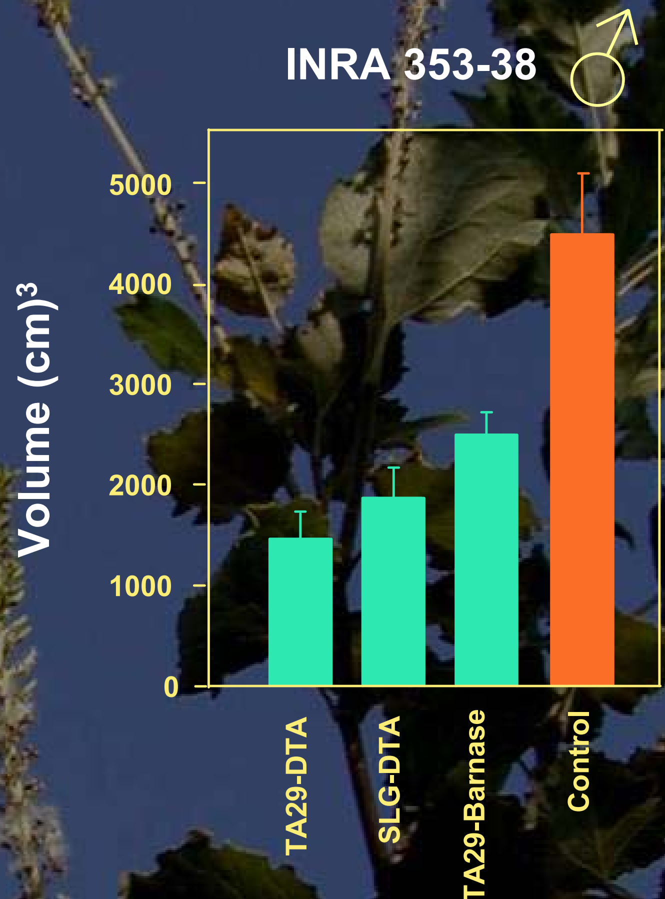
REALITY

- Floral <u>predominant</u> rather than floral specific expression
- Need to <u>mitigate</u> (attenuate) deleterious vegetative side effects

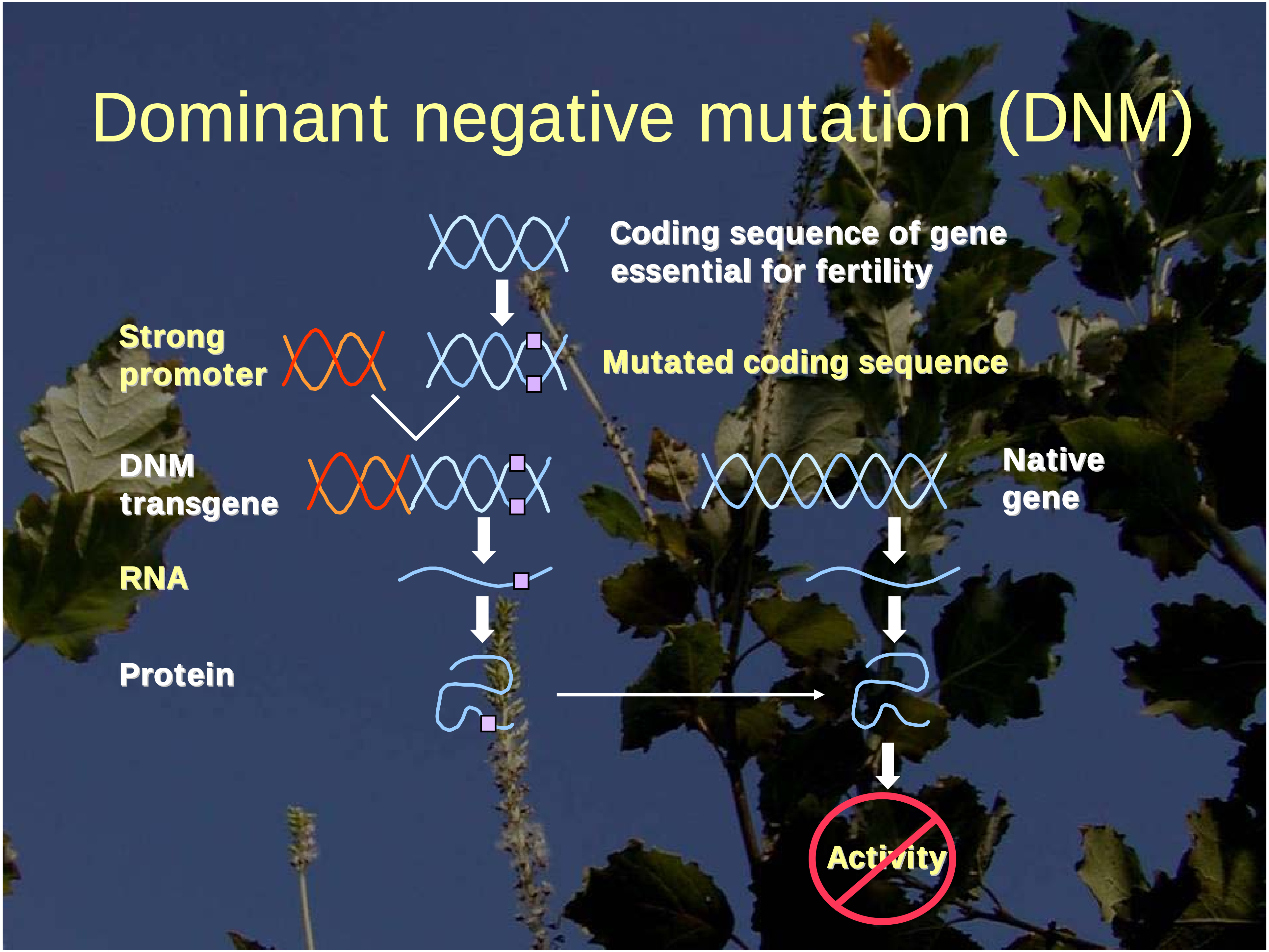
Ablation genes can have subtle effects on plant vigor

Impaired early growth of field grown transgenic poplars (1.5 years of growth, volume = ht x diameter²)



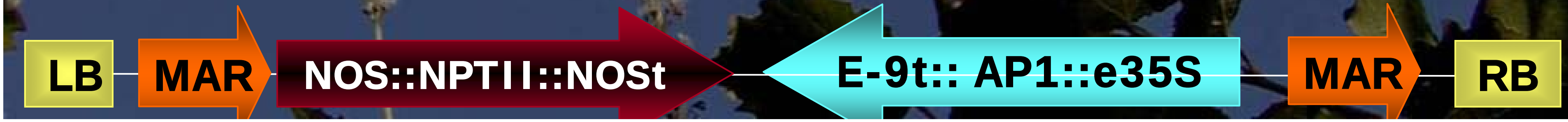


TA29, tapetal; SLG, self-incompatibility locus G; TTS, transmitting-tissue specific; Barnase, *Bacilllus* RNAse; DTA, ribosomal toxin


Dominant negative mutation (DNM)
Coding sequence of gene essential for fertility
Strong promoter
Mutated coding sequence
DNM transgene
Native gene
RNA
Protein
Activity

Arabidopsis DNM transgenes

- All transgenes are variants of *AP1* and *AG*
  - Site-directed mutagenesis used to alter specific amino acids in the MADS domain
    - 3 different constructs for *AP1* and *AG*
  - Truncation of *AP1* transcription activation domain
- Evaluated in transgenic Arabidopsis
  - Selected most promising constructs for testing in poplar



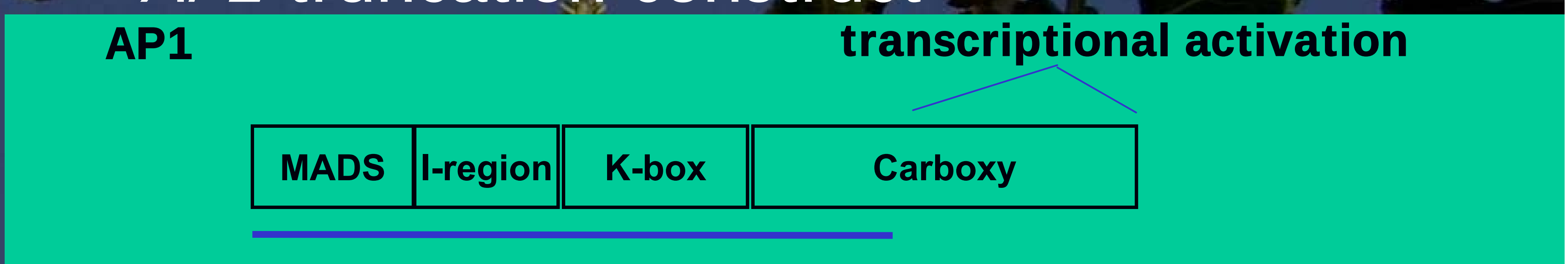
7 DNM transgenes

- Site-directed mutagenesis Arabidopsis genes alter amino acids in MADS domain

```
AP1   GRGRVQLKRIENKINRQVTFSKRRAGLLKKAHEISVLCDAEVALVVFSHKGKLFEY
 AG   ...KIEI.....TT......C...N......Y.L..........I...SR.R.Y..

M1     T AG
M2                             EE
M3                      LE
```

- *AP1* truncation construct



Phenotypes induced by DNM transgenes

- 6 DNM transgenes analyzed to date
  - Double enhancer 35S promoter
- Induced mutant floral phenotypes at high frequency
  - 34-96% of Arabidopsis T1 transgenics
  - Mutants usually do not set seed
- DNM phenotypes often novel
  - May have partial gain-of-function or loss-of-function characteristics

AG DNM transgenic phenotypes


Wild-type


AG-m1


AG-m2

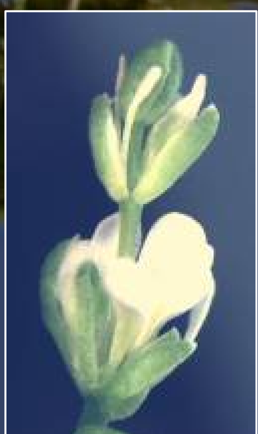
AG-m3


AP1 DNM transgenic phenotypes

Wild-type

AP1-6T line15

AP1-m3



AP1-6T line14

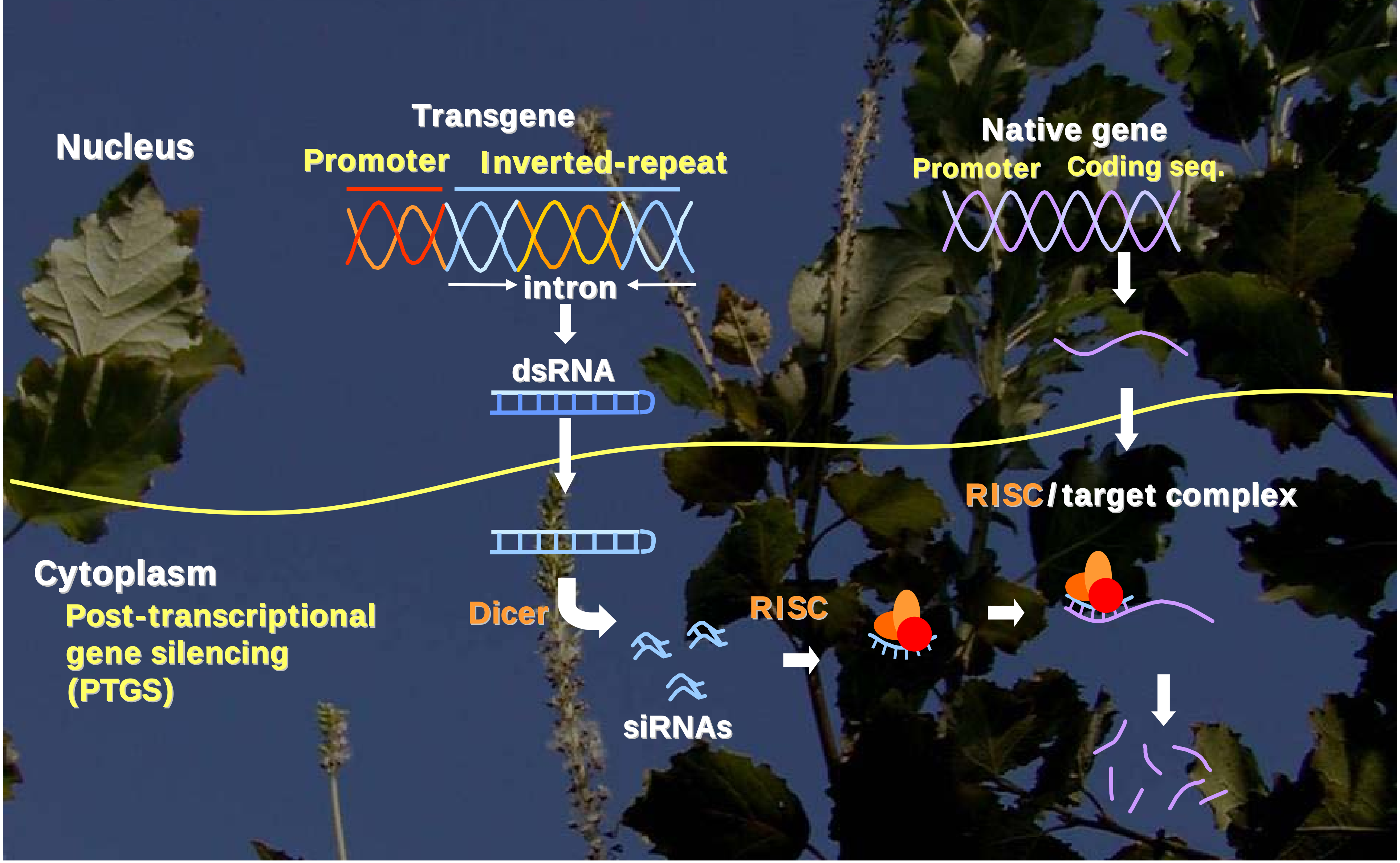
AP1-m1

Arabidopsis DNM T1 transgenic summary

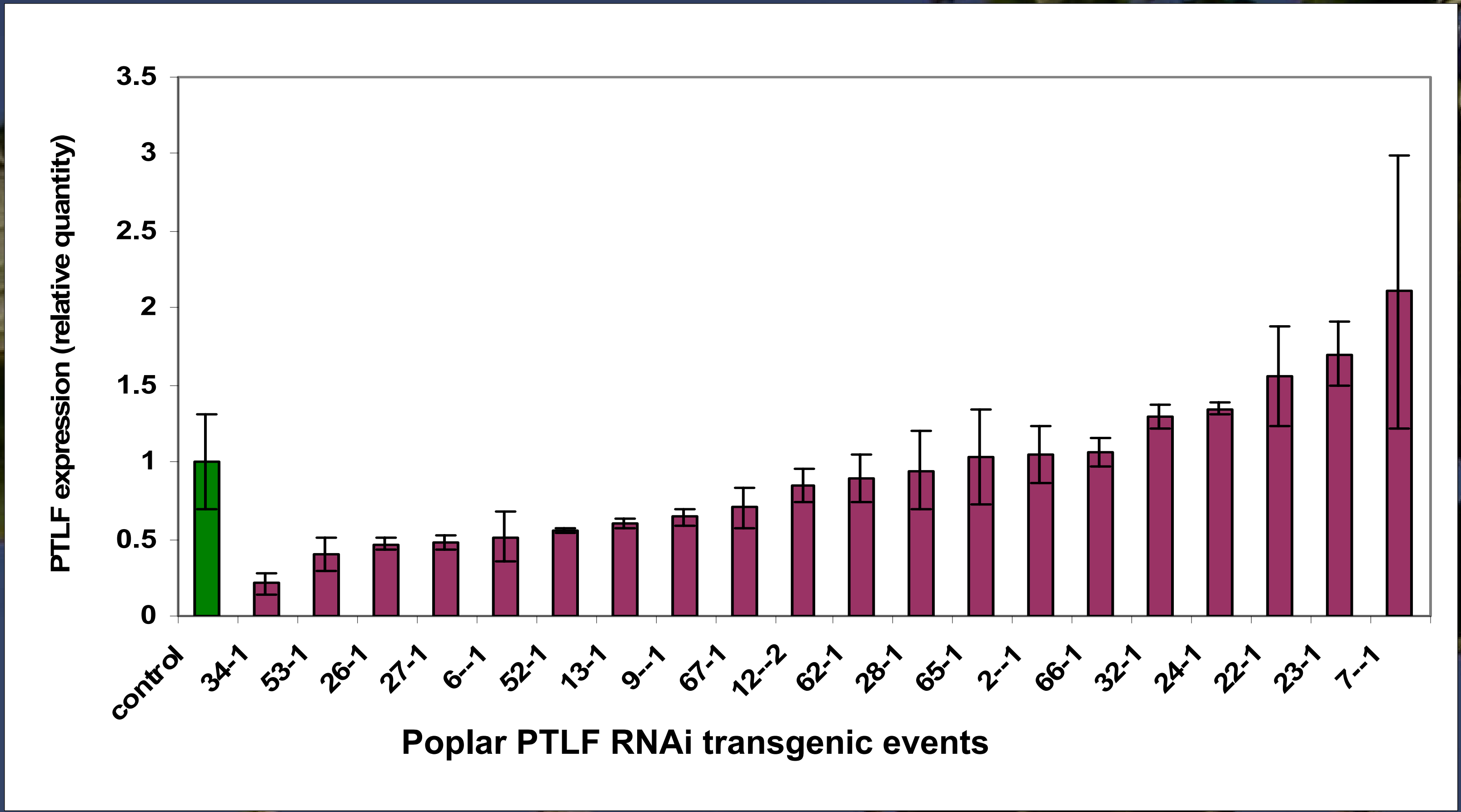
Transgene	No. T1 lines	No. mutants	No. sterile lines*	General floral phenotype
AP1-6T	40	37	37	Gain
AP1-m1	32	11	11	Gain
AP1-m2	20	8	7	Loss
AP1-m3	18	9	6	Loss
AG-m1	25	21	9	Gain
AG-m2	21	10	6	Loss
AG-m3	30	19	9	Loss

*Additional mutant lines have reduced fertility

RNA interference (RNAi)
(gene suppression, gene silencing)

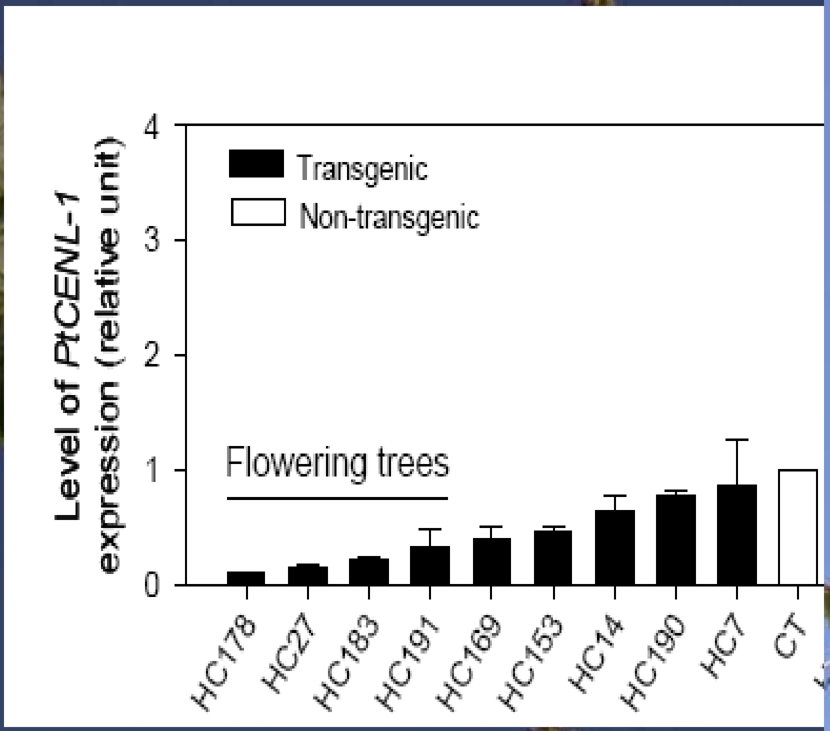


Variation in *PTLF* gene expression in vegetative tissues among *PTLF*-RNAi transgenic events



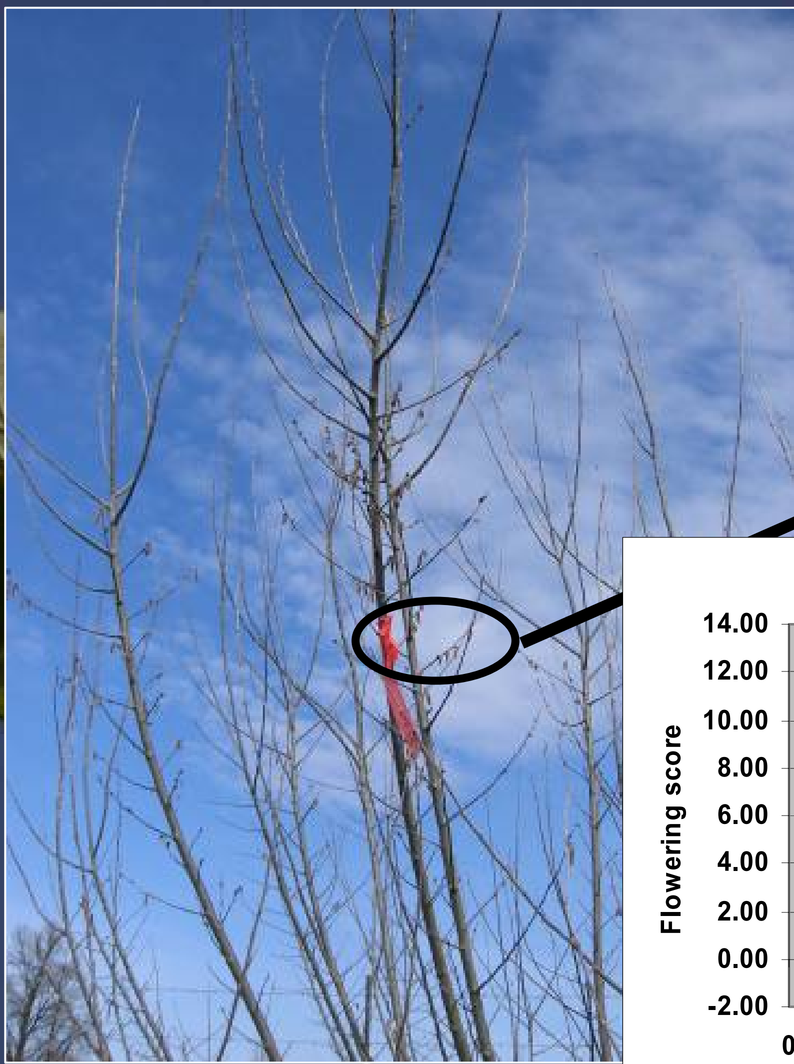
- **Each bar represents 4-5 ramets per event**
  Error bars are standard deviation over 2 technical PCR replicates

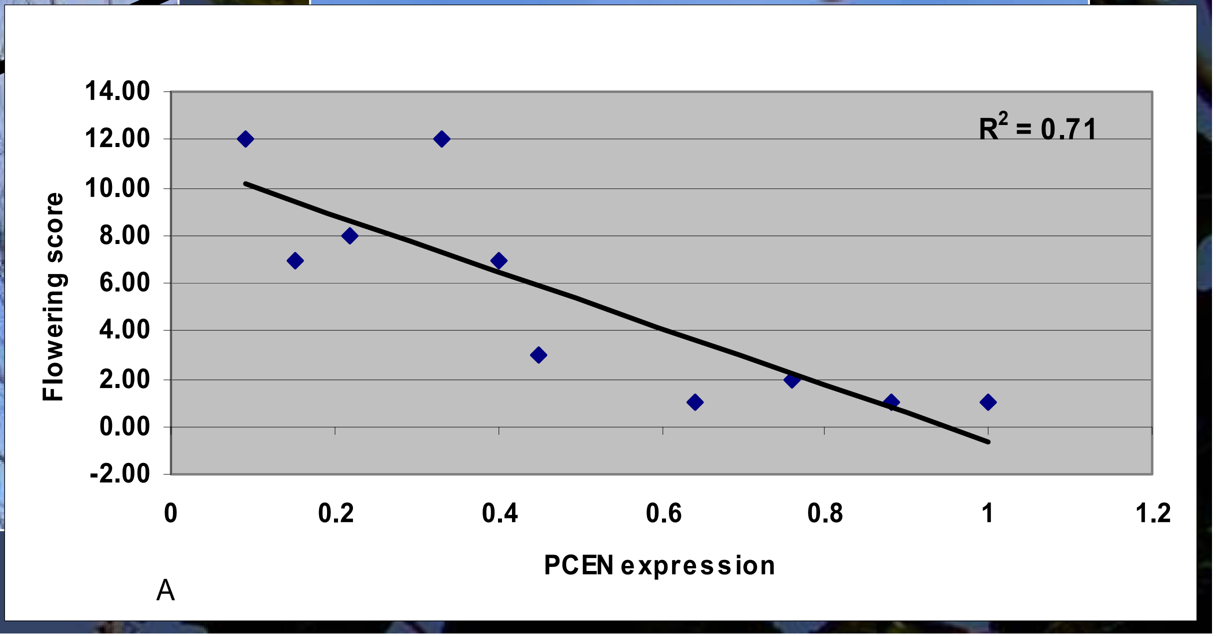
Gene suppression predicts flowering from RNAi of *PCENL-1* in poplar



Event 191-flowering in the field at 2 years

The most strongly suppressed events flowered most strongly in Spring 2006





Many types of RNAi sterility constructs

Analysis in three poplar clones

12 single or two gene constructs

4 double RNAi constructs

1 Triple: PTAP / PTLF / PTAG

Field testing of RNAi sterility constructs

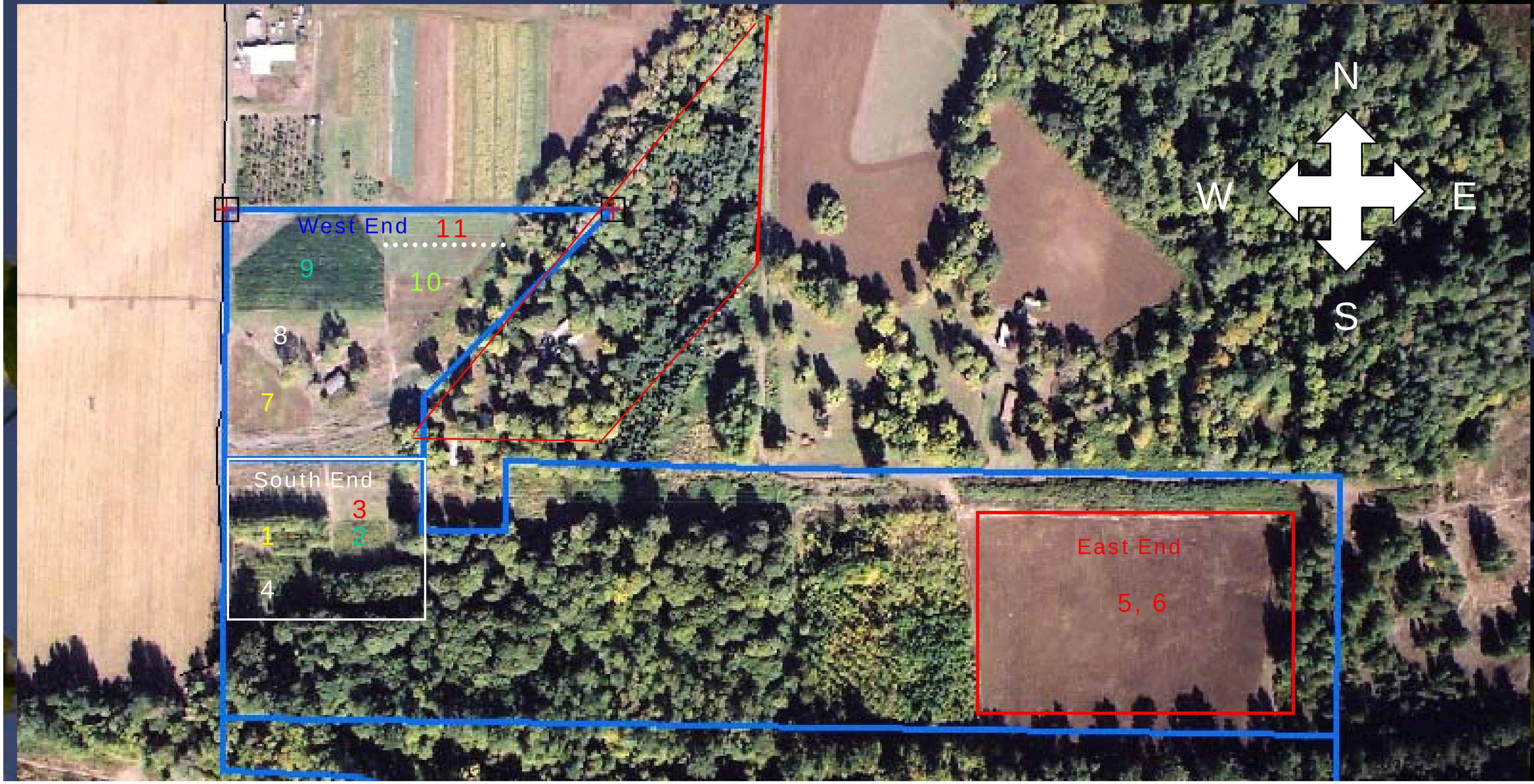
Construct name	Poplar clones with PCR positive events		
	717	353	6K10
PTD-IR	22	22	27
PTAG-IR	24	24	22
PTAG-IRwMAR	-	-	13
PTLF-IR	25	21	21
PTAP-IR	20	20	30
35S:PTLF-IR/35S:PTAG-IR	21	27	24
PTAP:PTAG-IR	24	28	15
PTAP:PTLF-IR	22	22	17
PTLF:PTAG-IR	26	18	11
Triple-IR	4	13	29
Han-PCEN	15 in field	-	27
Han-PMFT	15 in field	-	21
PAGL24-IR	22	12	13
PAGL20-IR	24	6	15
PFT-IR	20	17	4
PFPFL1-IR	5	In selection	-
PFPFL2-IR	20	5	20
PFT-IR:PAGL20-IR	48	-	-
PFT-IR:PAGL20-IR+PFPFL1-I	7	7	-

Field testing of non-RNAi based constructs

Construct name	Poplar clones with PCR positive events		
	717	**353**	**6K10**
Overexpression			
35S:PMFT	19 in field	-	24
35S:PCEN	19 in field	27	22
35S:PSVPL	32	29	39
35S:PAGL24	30	27	24
DNM(w/MAR)			
En35S:AG-m3	22	28	17
En35S:AG-m2	17	7	1
En35S:AP1-m3	27	31	25
En35S:AP1-m2	20	13	3

Total events = 1,440

Field sites (red)



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- National Science Foundation Industry/University Cooperative Research Center
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    - Arborgen
    - Boise Cascade
    - Potlatch
    - Weyerhaeuser


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