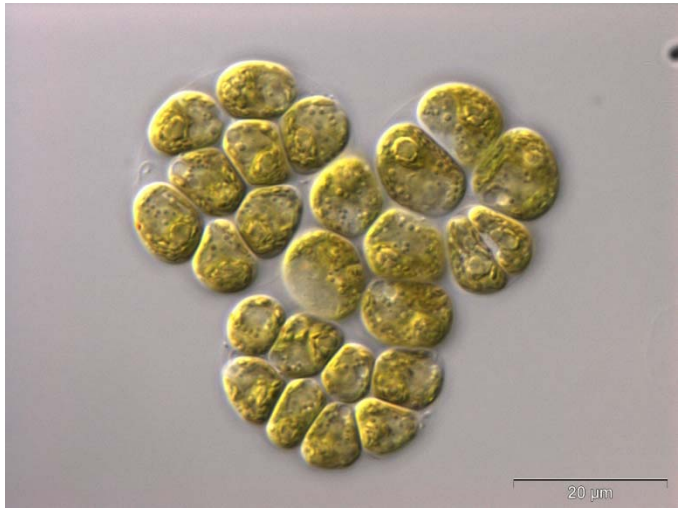
Transgenic Microalgae – Problems and Perspectives



Michael Schroda

Max Planck Institute
of Molecular Plant Physiology

Transgenic Microalgae – Problems and Perspectives

- Rationale – why do we want to genetically manipulate microalgae?
- Strategy for microalgal transfection (transformation)
- Overview to transformed microalgae

  Diatoms

  Thalassiosira pseudonana

  Phaeodactylum tricornutum

  Rhodophytes (red algae)

  Cyanidioschyzon merolae

  Chlorophytes

  Chlorella

  Haematococcus pluviales

  Dunaliella salina

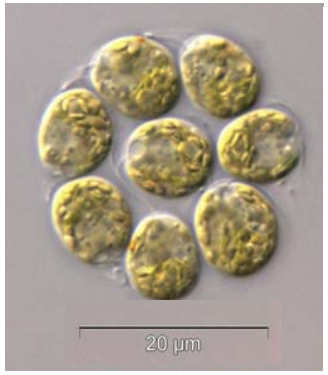
  Chlamydomonas reinhardtii (*Volvox carteri*)
- Strategies for targeted knock-out / knock-down in *Chlamydomonas reinhardtii*

  knock-out libraries

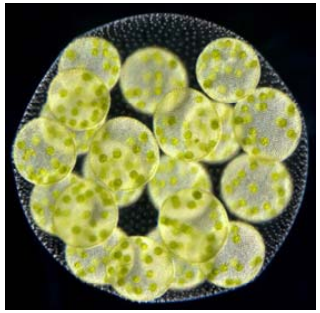
  (inducible) amiRNAs
- Overcoming problems in gene expression by targeted chromatin remodeling
- Summary & Outlook

Why transform microalgae?

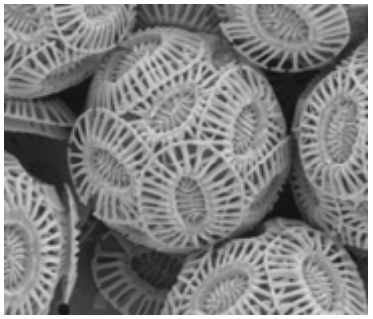
More and more microalgal genome sequences are completed:



Chlamydomonas reinhardtii
(Chlorophytes)

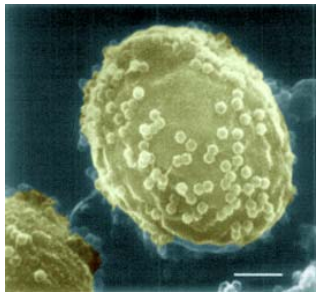


Volvox carteri
(Chlorophytes)

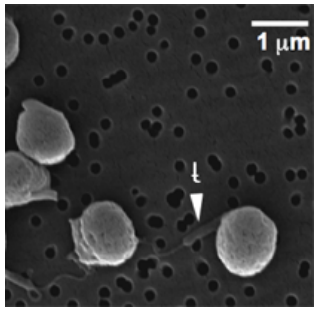


Emiliania huxleyi
(Coccolithophores)

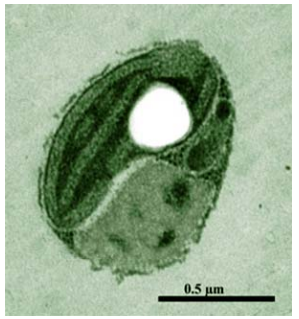
Cyanidioschyzon merolae
(Rhodophytes)



Chlorella variabilis
(Chlorophytes)



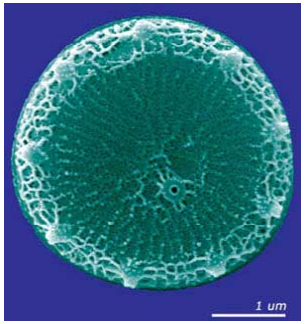
Micromonas pusilla
(Prasinophytes)



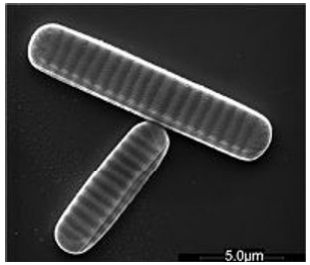
Ostreococcus lucimarinus / tauri
(Prasinophytes)

Guillardia theta & Bigelowiella natans
(Cryptomonads)

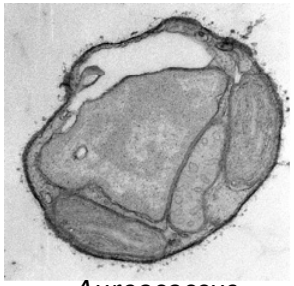
Phaeodactylum tricornutum
(Diatoms)



Thalassiosira pseudonana
(Diatoms)



Fragilariopsis cylindrus
(Diatoms)



Aureococcus
anophagefferens
(Pelagophytes)

Source: mainly JGI

Why transform microalgae?

Basic research

- Photosynthesis (*Chlamydomonas reinhardtii*)
- Cilia/flagella (*Chlamydomonas reinhardtii*)
- Development of multicellularity (*Volvox carteri / Chlamydomonas reinhardtii*)
- Primary and secondary endosymbiosis (Rhodophytes, Diatoms, Cryptophytes)
- Extremophiles (*Cyanidioschyzon merolae*, *Chlamydomonas nivalis/acidophila*)

Ecological importance

- ~50% of annual carbon fixation by microalgae
- Algal blooms (*Aureococcus anophagefferens*)

High-end commercial products

- Carotenoids (*Haematococcus*, *Dunaliella*)
- Feed stock for aquaculture (*Nannochloropsis*)
- Food supplementals (*Chlorella*)
- Hydrogen (*Chlamydomonas reinhardtii*)
- Biodiesel (*Botryococcus braunii*)

Why transform microalgae?

Metabolic engineering

Reduce expression of algal genes

knock-out; knock-down by expressing antisense, inverted repeats, amiRNAs

→ eliminate pathways competing with that leading to desired product

Express foreign genes

→ generate strains with new biosynthetic properties

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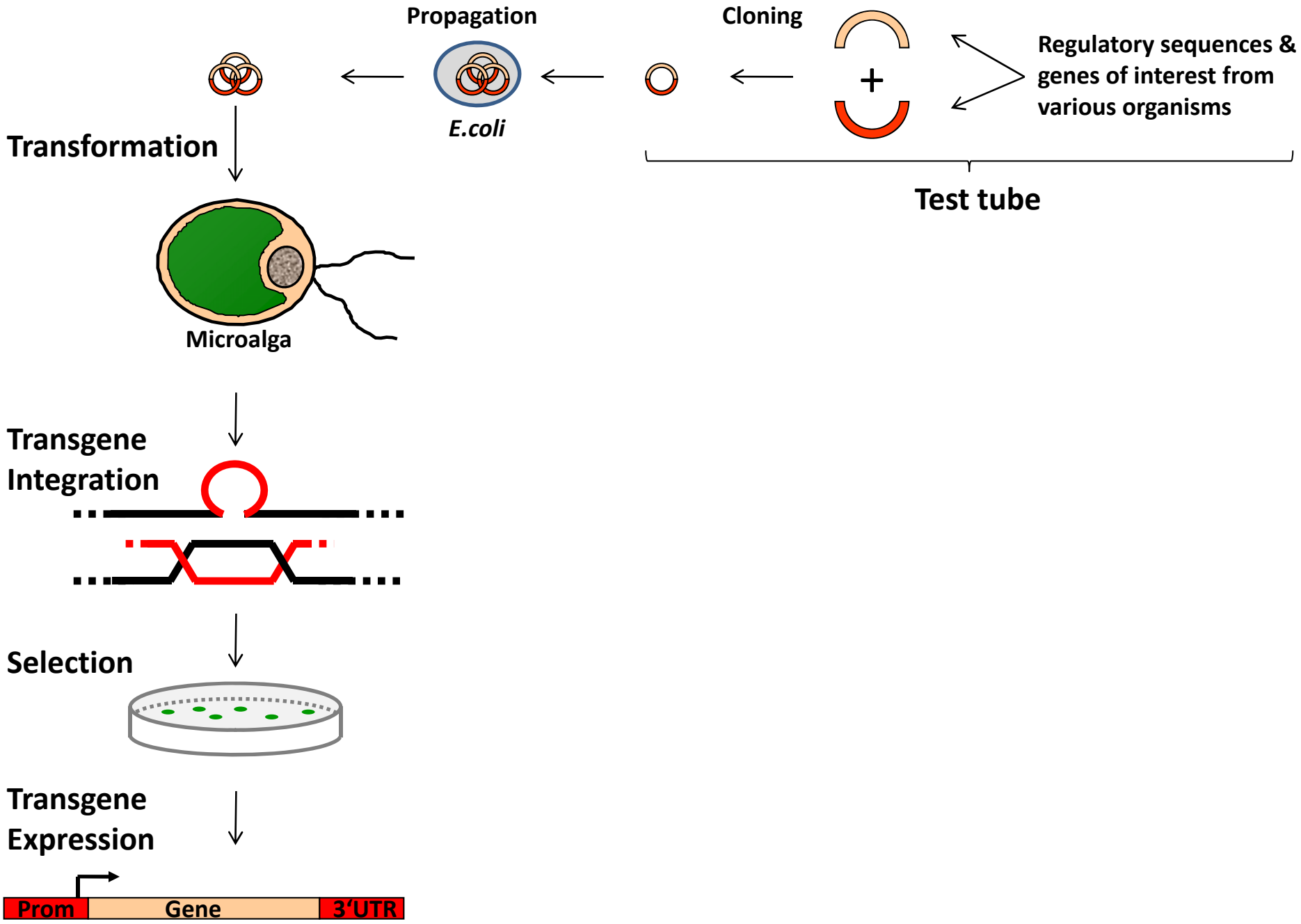
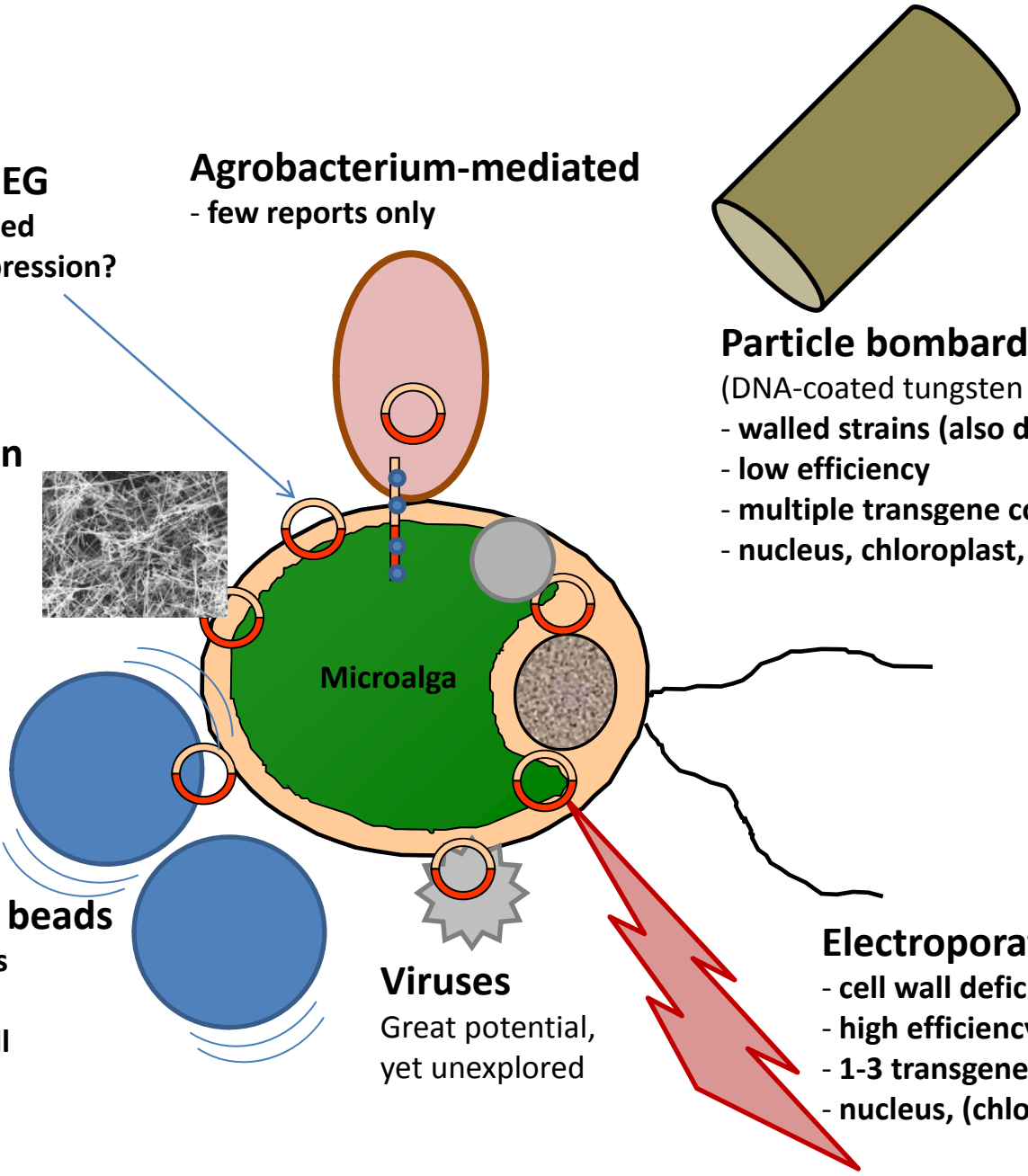
Strategie for microalgal transformation



Transformation techniques for microalgae



Incubation with PEG
- real protoplasts needed
- only for transient expression?

Agrobacterium-mediated
- few reports only

Particle bombardment
(DNA-coated tungsten particles; helium gun)
- walled strains (also diatoms!)
- low efficiency
- multiple transgene copies/cell, concatemers
- nucleus, chloroplast, mitochondria

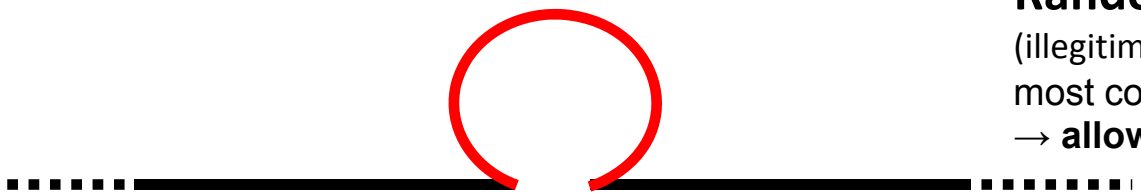
Agitation with silicon carbide whiskers
- walled strains
- medium efficiency
- nucleus

Agitation with glass beads
- cell wall-deficient strains
- medium efficiency
- 1-3 transgene copies/cell
- nucleus, (chloroplast)

Viruses
Great potential,
yet unexplored

Electroporation
- cell wall deficient strains
- high efficiency (after optimization...)
- 1-3 transgene copies/cell
- nucleus, (chloroplast)

Transgene Integration



Random integration

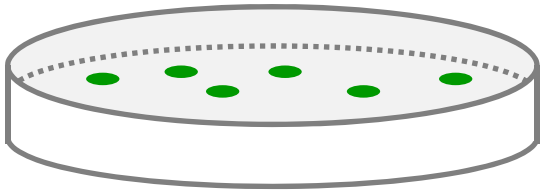
(illegitime recombination, non-homologous recombination)
most common in algal nuclei
→ **allows generation of knock-out libraries**

Homologous recombination

usually rare in nuclei, most common in organelles
→ **allows targeted knock-out / gene manipulation**

Transient, no stable integration

Selection



Complementation of auxotrophic strains (recessive selectable markers)

- Generate strains by random mutagenesis (e.g. UV) that cannot synthesize essential metabolite
- Grow cells by exogenously supplementing essential metabolite
- Transform cells with wild-type gene
- Select for transformants by plating cells on medium lacking essential metabolite

(e.g.: defects in nitrate reductase, grow cells on ammonium, transform with *NR* gene, select on nitrate

defect in argininosuccinate lyase, grow with arginine, transform with *ARG7* gene select on medium lacking arginine

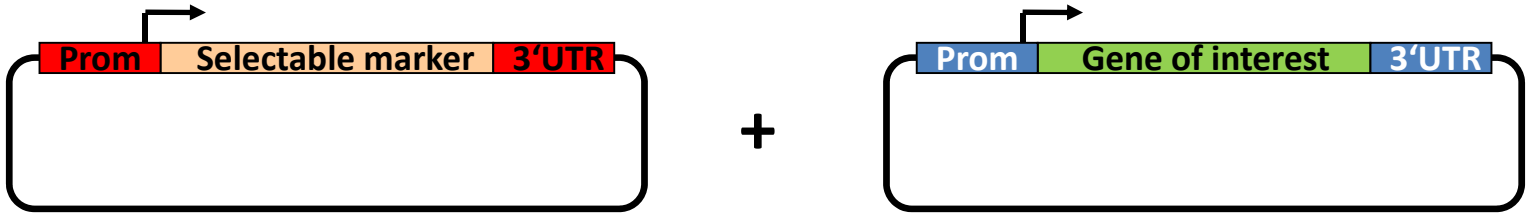
Introducing resistance gene to antibiotic or herbicide (dominant selectable markers)

- Transform cells with resistance gene (usually under control of homologous promoters)
- Select for transformants by plating cells on medium containing antibiotic / herbicide

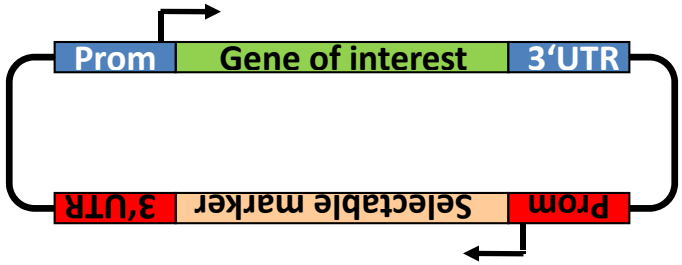
(e.g.:	*ble*	resistance to	phleomycin antibiotics (zeocin)
	aadA		spectinomycin, streptomycin
	aphVIII		paromomycin
	aph7“		hygromycin B
	nat		nourseothricin
	nptII		kanamycin
	bar		phosphinothricin (herbicide)

Transgene Expression

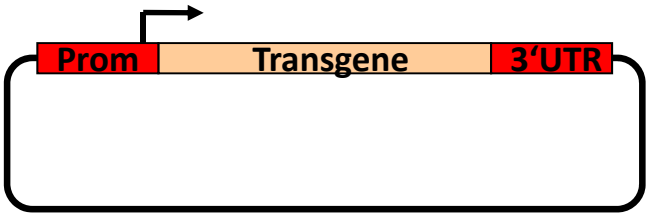
Co-transformation with 2 separate plasmids for selectable marker and gene of interest
(20-50% of transformants contain construct with gene of interest)



Co-transformation with construct for selectable marker and gene of interest on one plasmid
(~80% of transformants contain construct with gene of interest)



Transgene Expression



Promoter

- Should be homologous promoter (from the algal species to be manipulated)
- Strong constitutive promoter (nuclear: *RBCS*, *FCP*; chloroplast: *psbA*)
- Inducible promoter (nuclear: nitrate reductase, carbonic anhydrase, cytochrome c_6)

Transgene

- Consider codon usage!
  (e.g. *Chlamydomonas* nuclear genes: G/C-rich, bias towards G/C in 3rd position
  chloroplast genes: A/T-rich)
- Introns (if genes in target alga are intron-rich, like in *Chlamydomonas reinhardtii*)

3‘UTR

- Should be homologous (from the algal species to be manipulated)
- Ensure presence of polyadenylation signal (transcript stability!)

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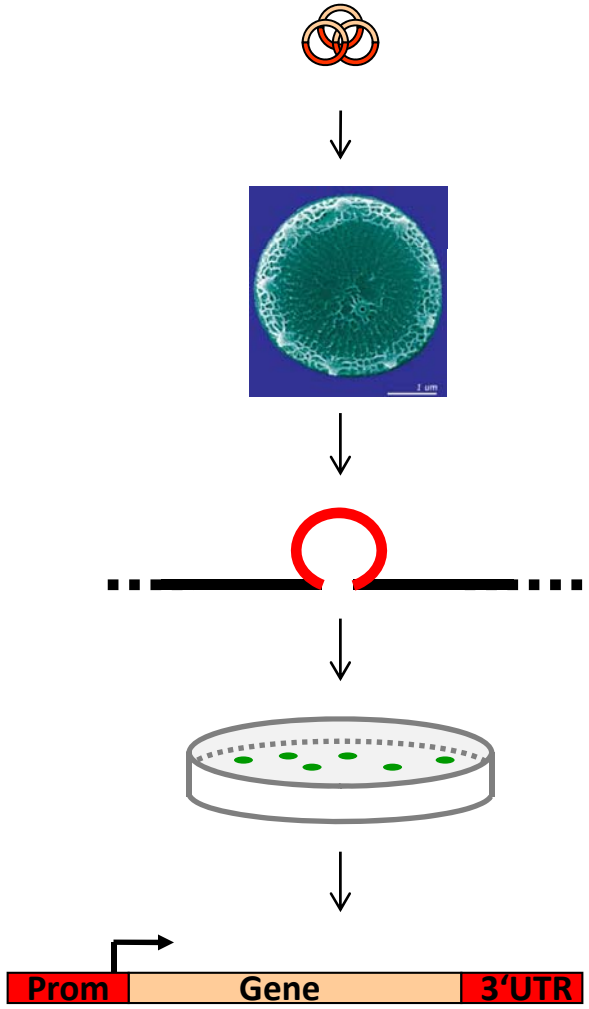


Transformation
Particle bombardment

Transgene Integration
Stable, random integration

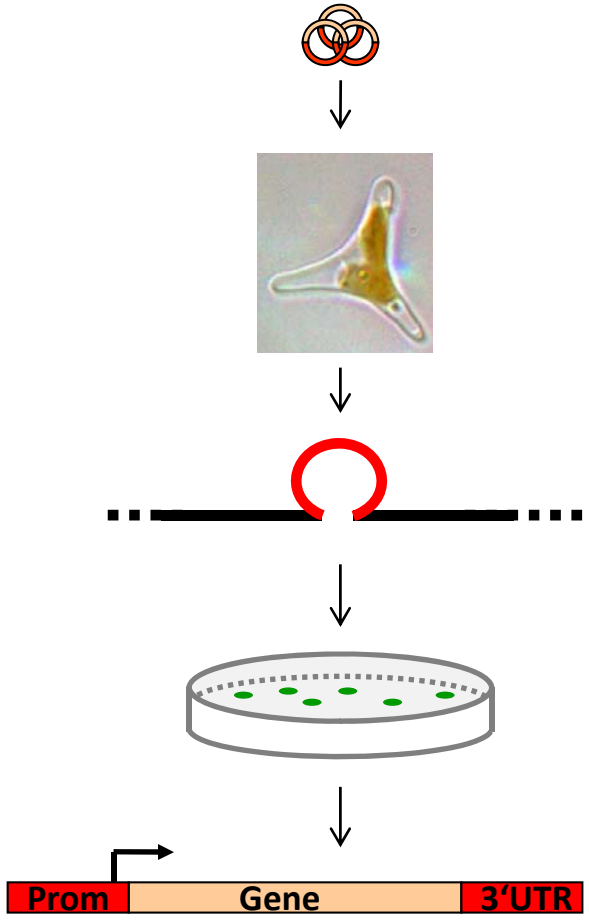
Selection
nat gene (nourseothricin resistance)

Transgene Expression
LHCF9 promoter (constitutive)
NR promoter (inducible)

Poulsen, N., Chesley, P. M., & Kroger, N. (2006) J Phycol 42, 1059–1065.

Phaeodactylum tricornutum

Second best established algal genetic system
But...
- Diploid in vegetative phase
- Particle bombardment: only low frequency
- No homologous recombination



Transformation
Particle bombardment

Transgene Integration
Stable integration

Selection
ble gene (resistance to phleomycin antibiotics)
nat gene (nourseothricin resistance)
nptII gene (kanamycin resistance)
sat-1 gene (streptothricin resistance)
cat (chloramphenicol resistance)

Transgene Expression
Promoters
Fucoxanthin chl-binding protein (*fcpA*)

Transgenes
GFP
Luciferase
uidA
Human Glut1
Chlorella HUP1
etc....
Inverted Repeat: RNAi!

Apt, K. E. et al. (1996) Mol. Gen. Genet. 252:572–579.
Zaslavskaia, L. A. et al. (2000) J. Phycol. 36:379–386.
Zaslavskaia, L. A. et al. (2001) Science 292:2073–2075.
Sakaguchi, T., Nakajima, K., & Matsuda, Y. (2011) *Plant Physiol*., in press.

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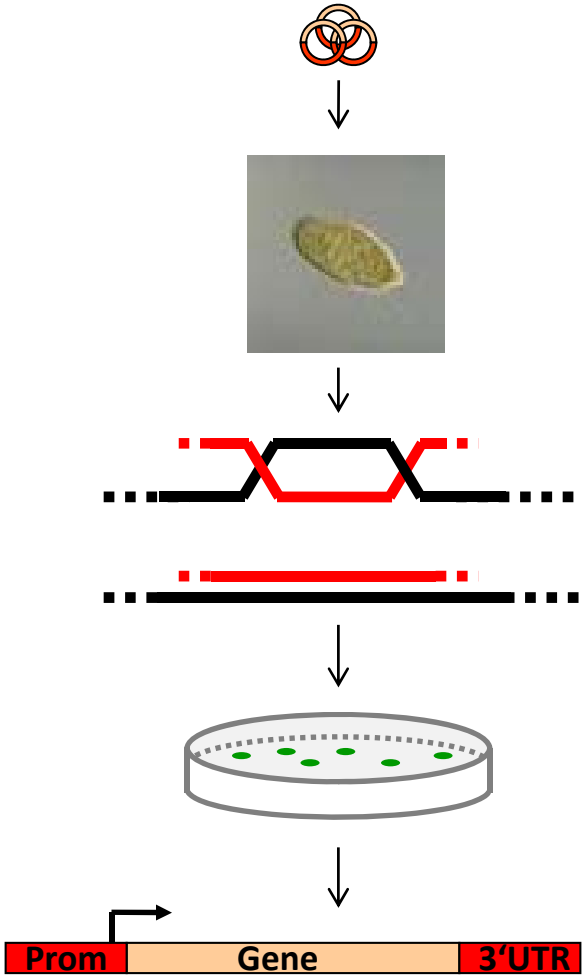
Cyanidioschyzon merolae



Transformation
Electroporation, PEG

Transgene Integration
Homologous recombination
Transient

Selection
GFP fluorescence
URA3, Complementation of uracil-requiring mutants
(selected initially for spontaneous mutants resistant to 5-fluoroorotic acid)

Transgene Expression

Promoters
URA3 promoter and
Catalase promoter
βTUB promoter

Transgenes
URA3 wild-type gene
antisense against catalase
GFP
HA-tagged β-tubulin

Minoda, A. et al. (2004) Plant Cell Physiol. 45:667–71.
Ohnuma, M. et al. (2008) Plant Cell Physiol. 49:117–120.
Ohnuma, M. et al. (2009) Protoplasma 236, 107-112.

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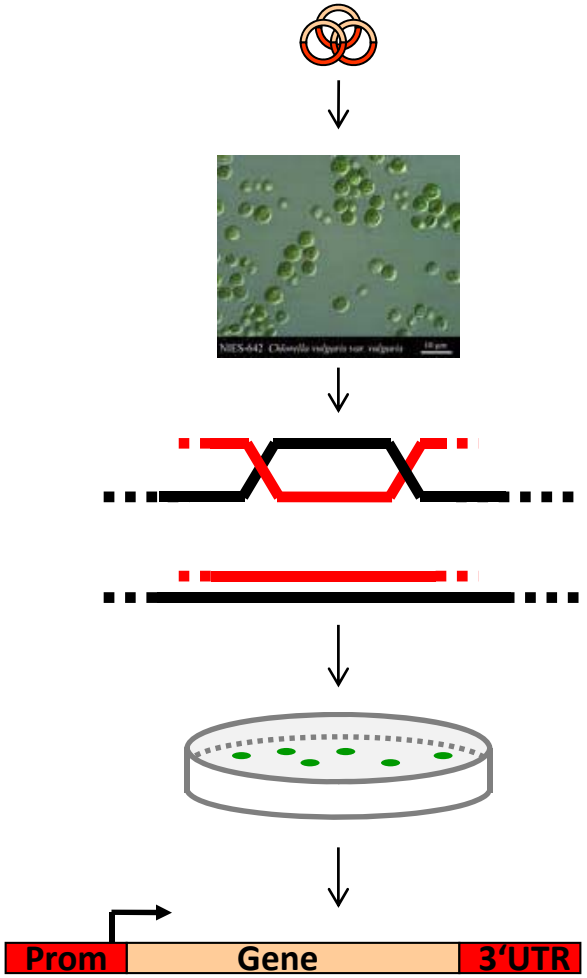
Chlorella (*C. ellipsoidea, C. saccharophila, C. sorokiniana. C. vulgaris, C. kessleri*)



Transformation

Particle bombardement, PEG, electroporation

Transgene Integration

Homologous recombination

Transient

Selection

none (...)

Complementation of *NR* gene mutant with wild-type *NR* gene

hpt (hygromycin resistance)

nptII (kanamycin resistance)

Transgene Expression

Promoters

plant CaMV-35S promoter

Chlorella virus promoter

Chlamydomonas RBCS2

Transgenes

Renilla luciferase

E. coli GUS

human growth hormone

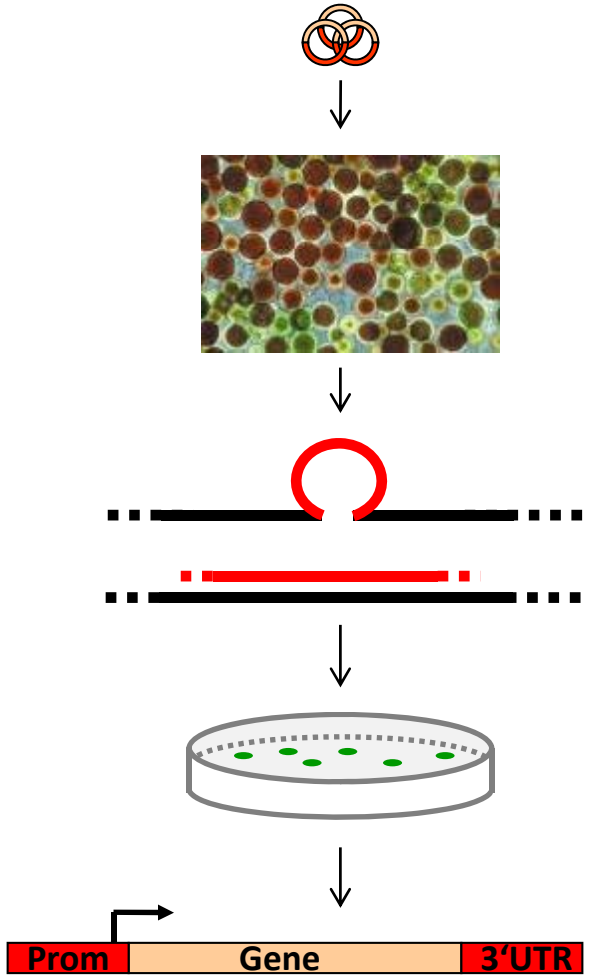
neutrophil peptide-1

flounder fish growth hormone

mosquito ovary peptide hormone

Jarvis, E. E. & Brown, L. M. (1991) Curr. Genet. 19:317–21.
Dawson, H. N. et al. (1997) Curr. Microbiol. 35:356–62.
Hawkins, R. L. & Nakamura, M. (1999) Curr. Microbiol. 38:335–41.
Borovsky, D. (2003) J. Exp. Biol. 206:3869–75.
Kim, D.-H. et al. (2002) Mar. Biotechnol. 4:63–73.

Haematococcus pluviales



Transformation
Particle bombardment, electroporation

Transgene Integration
Stable integration (concatemers?)
Transient

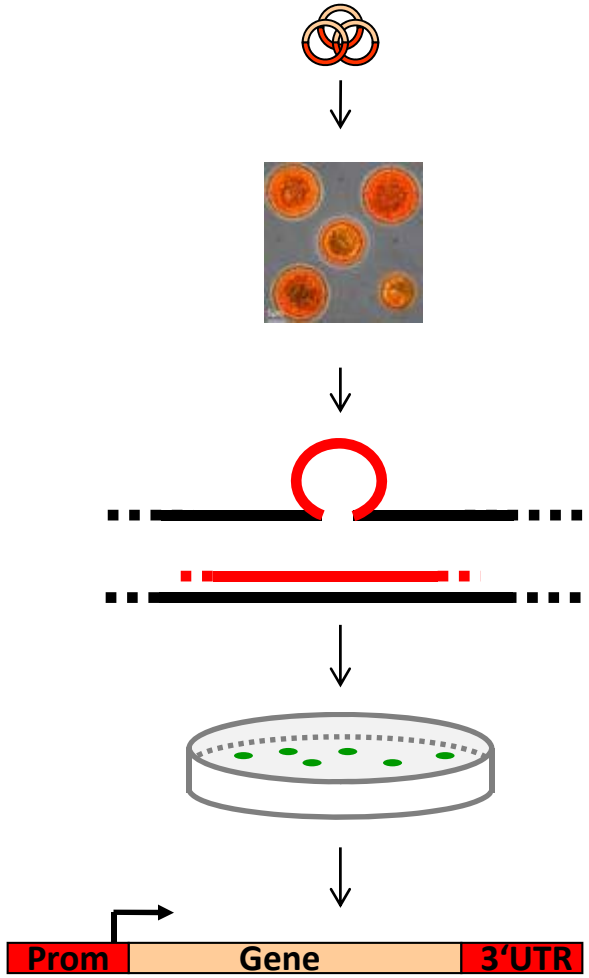
Selection
none (...)
Modified phytoene desaturase gene (norflurazon resistance)

Transgene Expression

Promoters	**Transgenes**
Viral SV40 promoter	*E. coli lacZ*
Phytoene desaturase promoter	and modified gene

Teng, C. et al. (2002) J. Appl. Phycol. 14:495-500.
Steinbrenner, J. & Sandmann, G. (2006) *Appl Environ Microbiol* 72, 7477-7484.

Dunaliella salina



Transformation

Particle bombardment, electroporation, glass bead agitation

Transgene Integration

Stable integration

Transient

Selection

none (...)

bar gene (resistance to herbicide phosphinothricin)

ble gene (resistance to phleomycin antibiotics)

(Transient) complementation of NR mutant

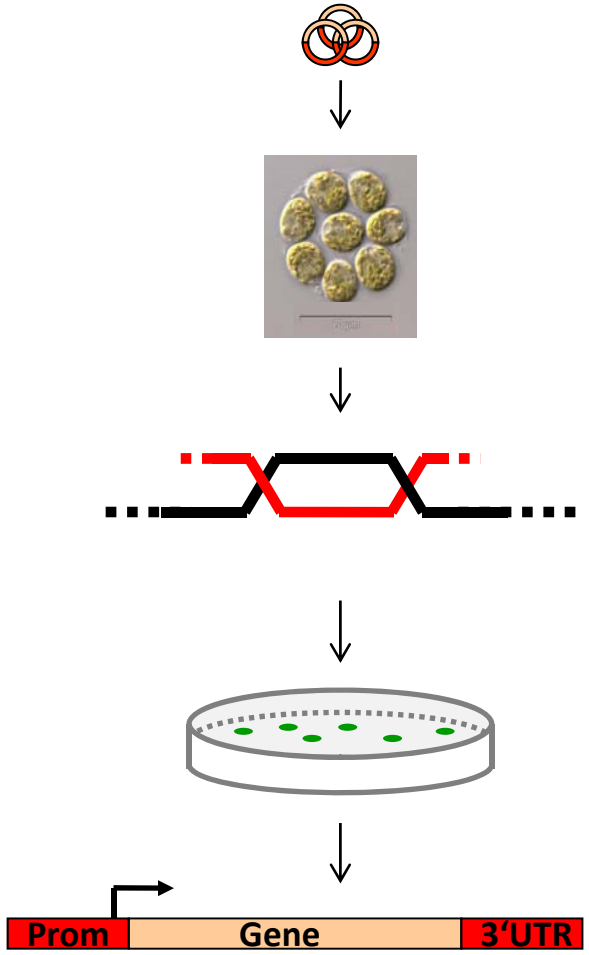
Transgene Expression

Promoters	**Transgenes**
Maize ubiquitin promoters	*EGFP*
CaMV-35S promoter	*GUS*
Chlamydomonas *RBCS2* promoter	Nitrate reductase
Actin promoter	
Carbonic anhydrase (salt-induced)	
Nitrate reductase (NO_3-induced)	

Geng DG et al. (2002) High Tech. Lett. 12:35–39.
Sun Y et al. (2005) Mol. Biotechnol .30:185–192.
Tan CP et al. (2005) J. Microbiol .43:361–365.
Geng DG et al. (2004) Acta Bot. Sin. 46:342–346.
Jiang GZ et al. (2005) Acta Gen. Sin. 32:424–433.
Li, J. et al. (2007) Gene 403, 132-142.
Li J et al. (2008) J. Appl. Phycol. 20:137–145.
Li, J. et al. (2010) Mol. Biol. Rep. 37, 1143-1154.

Chlamydomonas reinhardtii

Best established algal system for cp transformation
But...
- High level expression only in *psbA* mutant



Transformation
Particle bombardment (agitation with glass beads)

Transgene Integration
Homologous recombination

Selection
Complementation of cp gene mutants (e.g. *atpB* in FUD50)
aadA gene (spectinomycin, streptomycin resistance)
aphA-6 (kanamycin resistance)

Transgene Expression

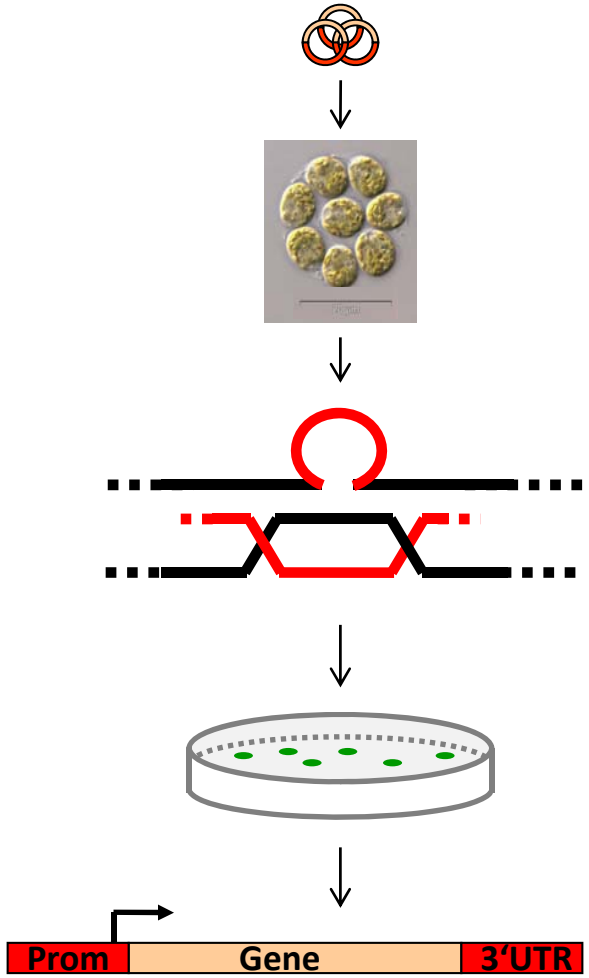
Promoters
psbA
atpA
rbcL
psbC
etc.

Transgenes
GFP
Luciferase
uidA
HSV8-scFv (single-chain antibody)
human therapeutic proteins
etc.

Boynton, J. E. et al. (1988) Science 240:1534–1538.
Goldschmidt-Clermont, M. (1991) *NAR* 19, 4083-4089.
Bateman, J. M. & Purton, S. (2000) Mol. Gen. Gen. 263:404–410.
Mayfield S.P et al. (2003) PNAS 100:438–442.
Mayfield, S. & Schultz, J. (2004) Plant J. 37:449–458.
Rasala, B. A. et al. (2010) Plant Biotechnol. J. 8:1–15.

Chlamydomonas reinhardtii

Best established algal system for nuclear transformation
But...
- No efficient homologous recombination
- No reliable high-level nuclear transgene expression



Transformation

Particle bombardment, electroporation, agitation with glass beads or silicon carbide whiskers, agrobacterium

Transgene Integration

Stable integration
Homologous recombination (ssDNA)

Selection

Complementation of auxotrophic strains (*arg7*, *nit1*, *atpC*, ...)
ble gene (resistance to phleomycin antibiotics)
aphVIII gene (resistance to paromomycin, kanamycin and neomycin
aph7" gene (resistance to hygromycin B)
aadA gene (spectinomycin, streptomycin resistance)

Transgene Expression

Promoters

HSP70A-RBCS2
PSAD
β_2*TUB*
NIT1 (inducible)
CYC6 (inducible)
etc...

Transgenes

GFP
Luciferase *(Gaussia, Renilla)*
Chlorella *HUP1*
antisense, Inverted Repeat
amiRNA!
etc...

Debuchy, R.,et al. (1989) *Embo J* 8, 2803-2809.
Fernandez, E. et al. (1989) *PNAS* 86, 6449-6453.
Kindle, K. L. (1990) *PNAS* 87, 1228-1232.
Stevens, D. R.et al. (1996) *Mol Gen Genet* 251, 23-30.
Fuhrmann, M. et al. (1999) *Plant J* 19, 353-361.
Schroda, M. et al. (2000) *Plant J* 21, 121-131.
Fuhrmann, M.et al. (2001) *J Cell Sci* 114, 3857-3863.
Sizova, I., et al. (2001) *Gene* 277, 221-229.
Molnar, A. et al. (2009) *Plant J* 58, 165–174.
Schmollinger, S. et al. (2010) *Curr Genet* 56, 383-389.

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- Overview to transformed microalgae

  Diatoms

  Thalassiosira pseudonana

  Phaeodactylum tricornutum

  Rhodophytes (red algae)

  Cyanidioschyzon merolae

  Chlorophytes

  Chlorella

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  Dunaliella salina

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  (inducible) amiRNAs
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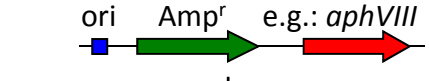
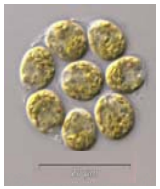
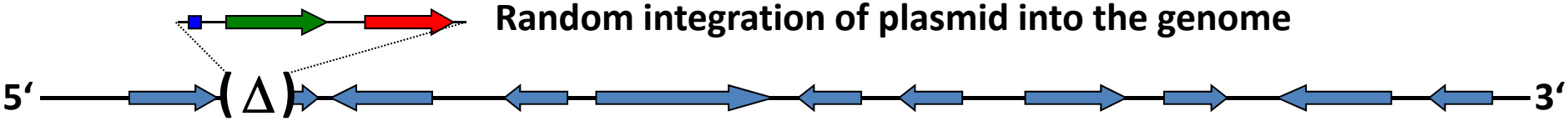
Insertional mutant libraries and their potential

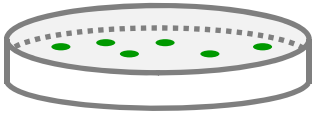


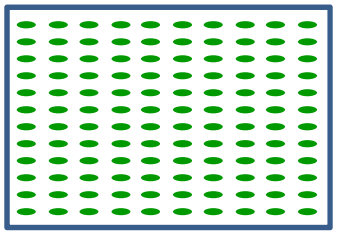


Random integration of plasmid into the genome



Select for resistance to paromomycin





Insertional mutant library (≥30.000)

Screen for specific mutant phenotype → Identify mutated gene(s) by plasmid rescue/ TAIL-PCR

Extract DNA, pool ‚rows‘ & ‚columns‘ → Screen for insertion in gene of interest by PCR

Challenges

- High transformation efficiency needed
- Screen for phenotype: vegetative cells need to be haploid
- Maintenance of thousands of transformants

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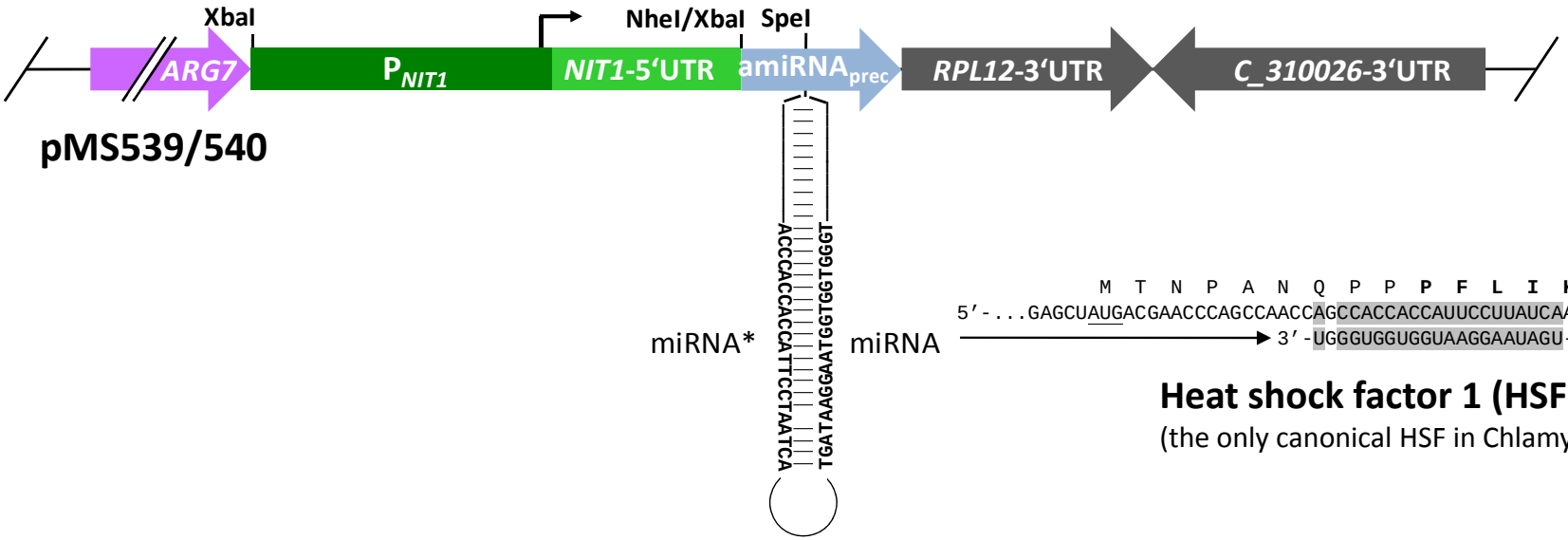
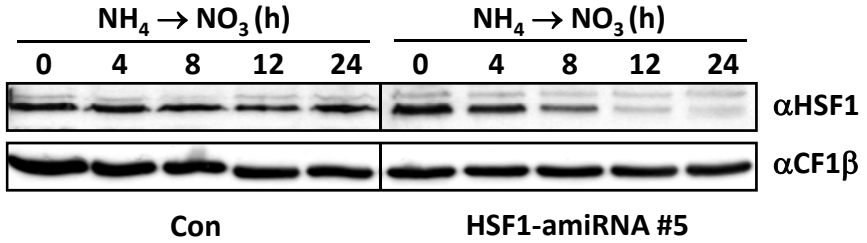
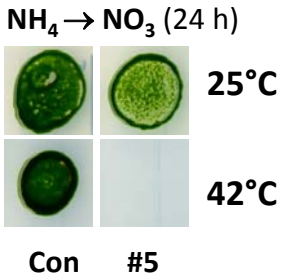
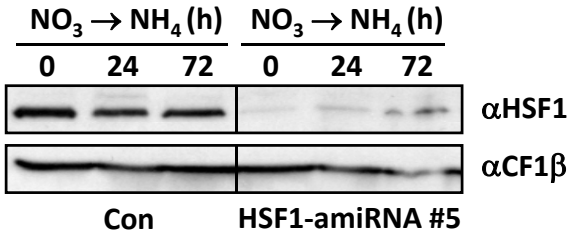
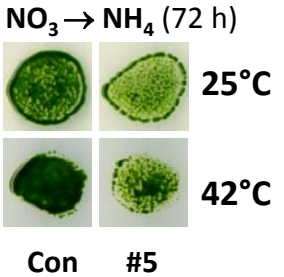
Establishment of a conditional amiRNA system for *Chlamydomonas* using heat shock factor 1 (HSF1) as target











Molnar, A. et al. (2009) *Plant J* **58,** 165–174; Schmollinger et al.,(2010) *Curr. Genet.*, 56, 383-389.

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The *HSP70A* promoter improves transgene expression

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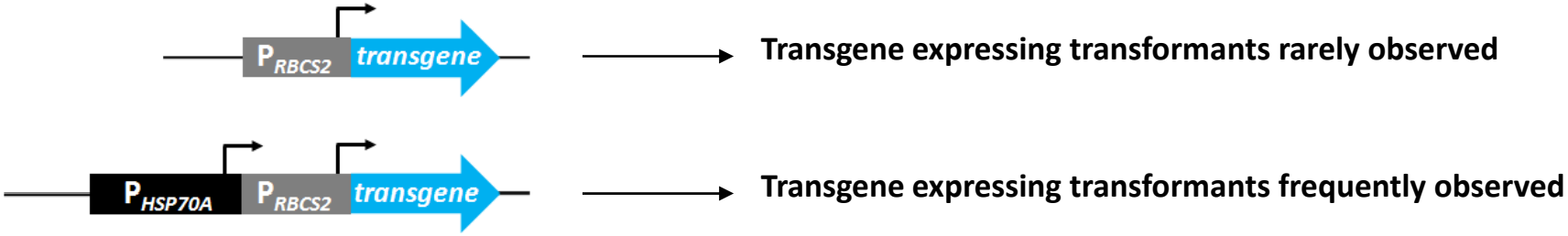
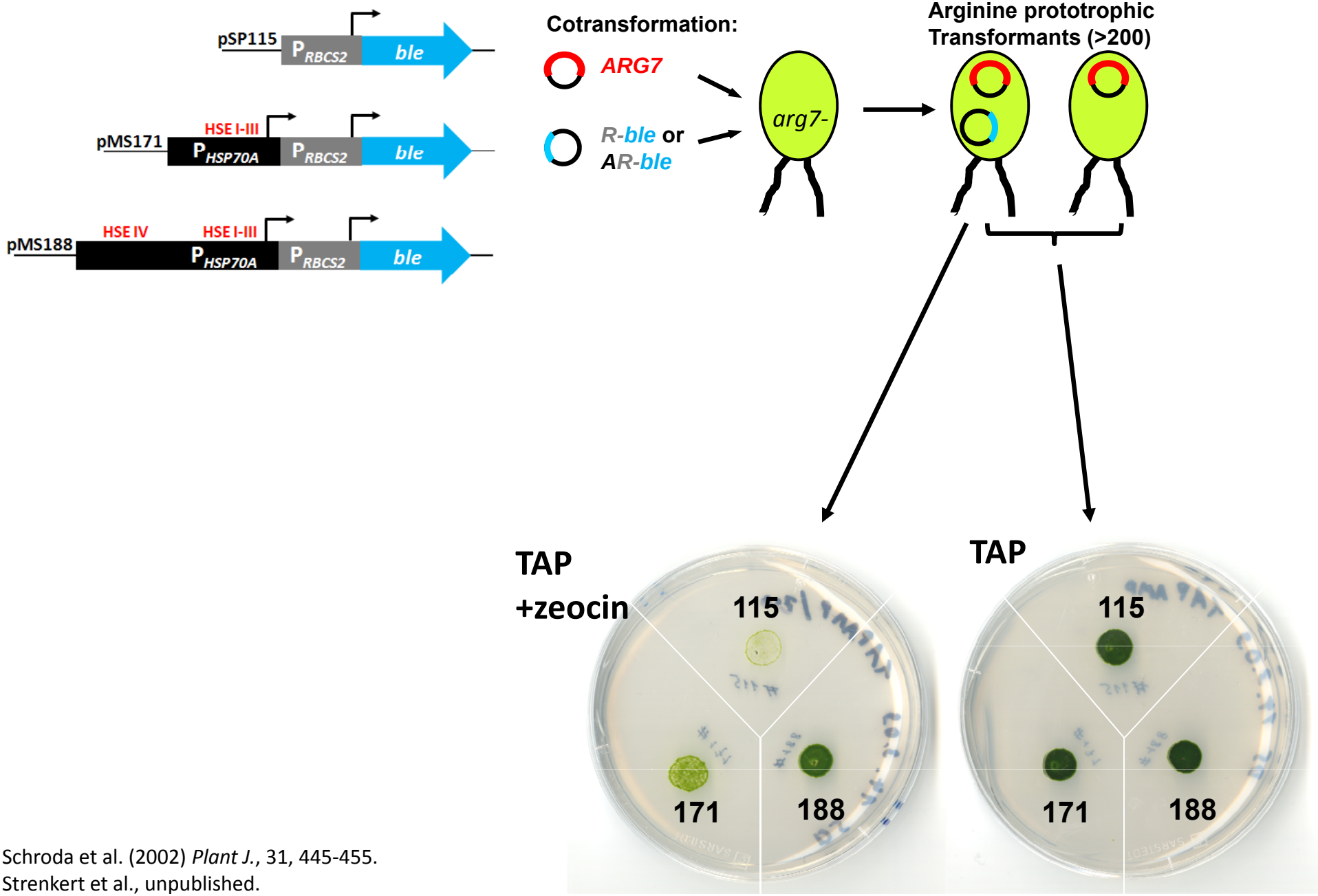


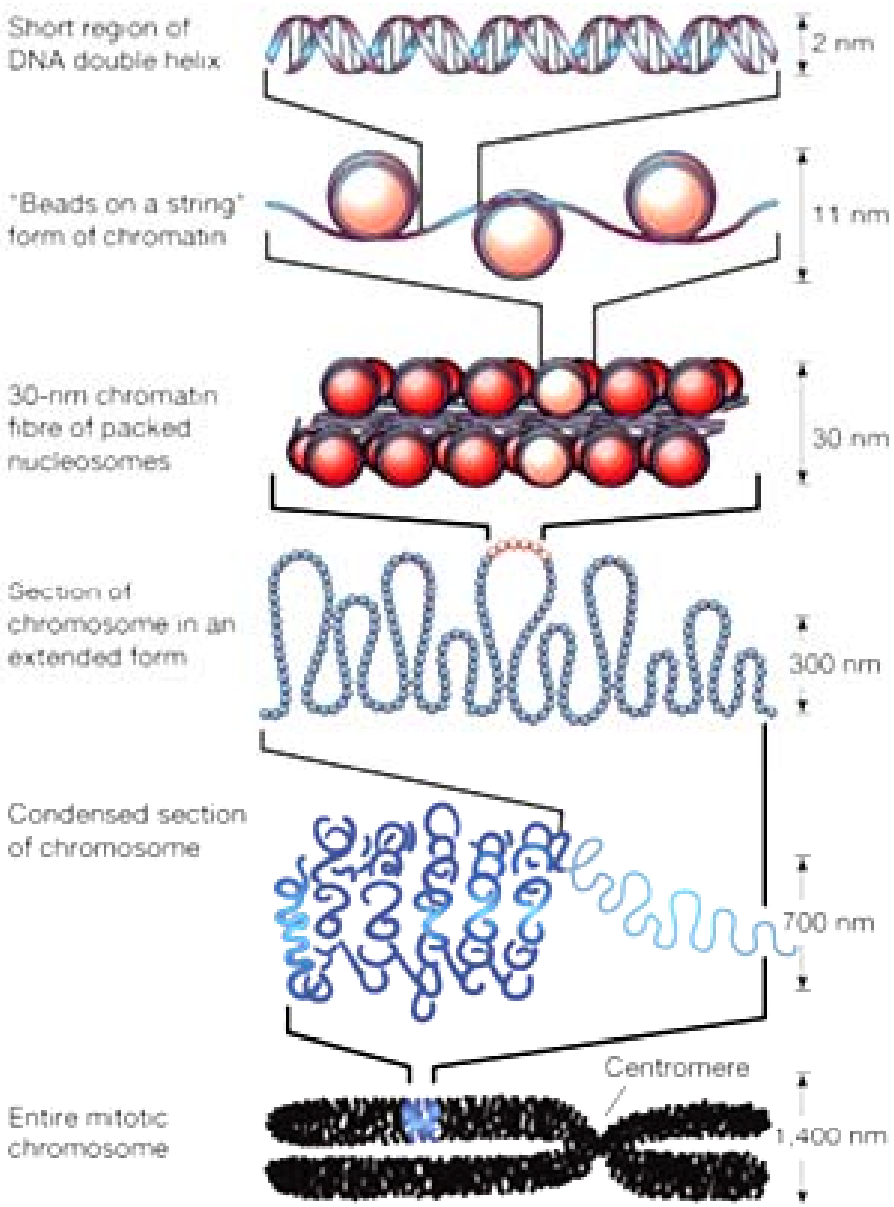
Schroda et al. (1999*) Plant Cell*, 11, 1165-78; Schroda et al. (2000) *Plant J.*, 21, 121-131.

The *HSP70A* promoter counteracts silencing of downstream promoters



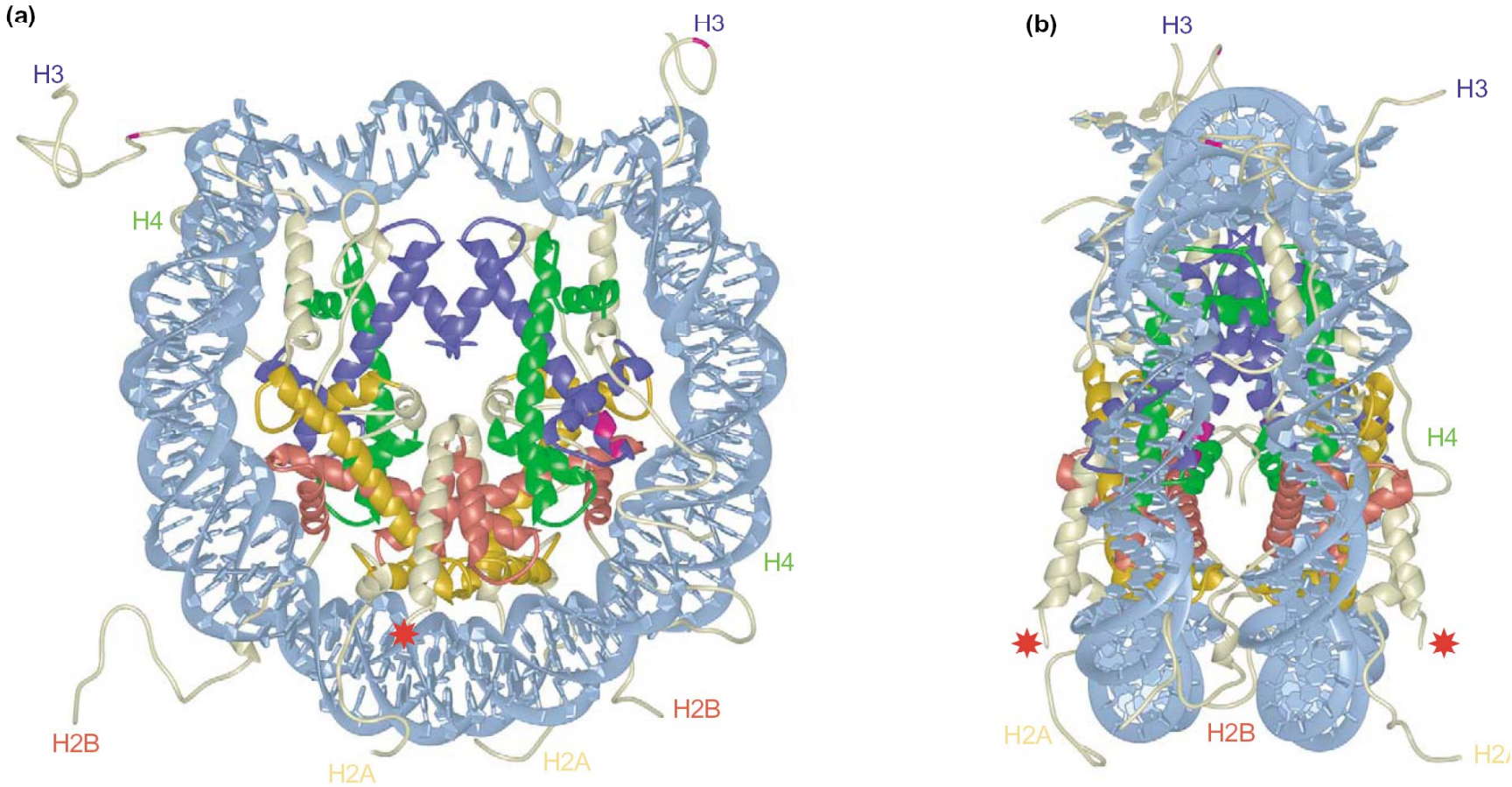
Schroda et al. (2002) *Plant J.*, 31, 445-455.
Strenkert et al., unpublished.

DNA is organized into chromatin



http://www.bric.dk/cms/upload/image_217.jpg

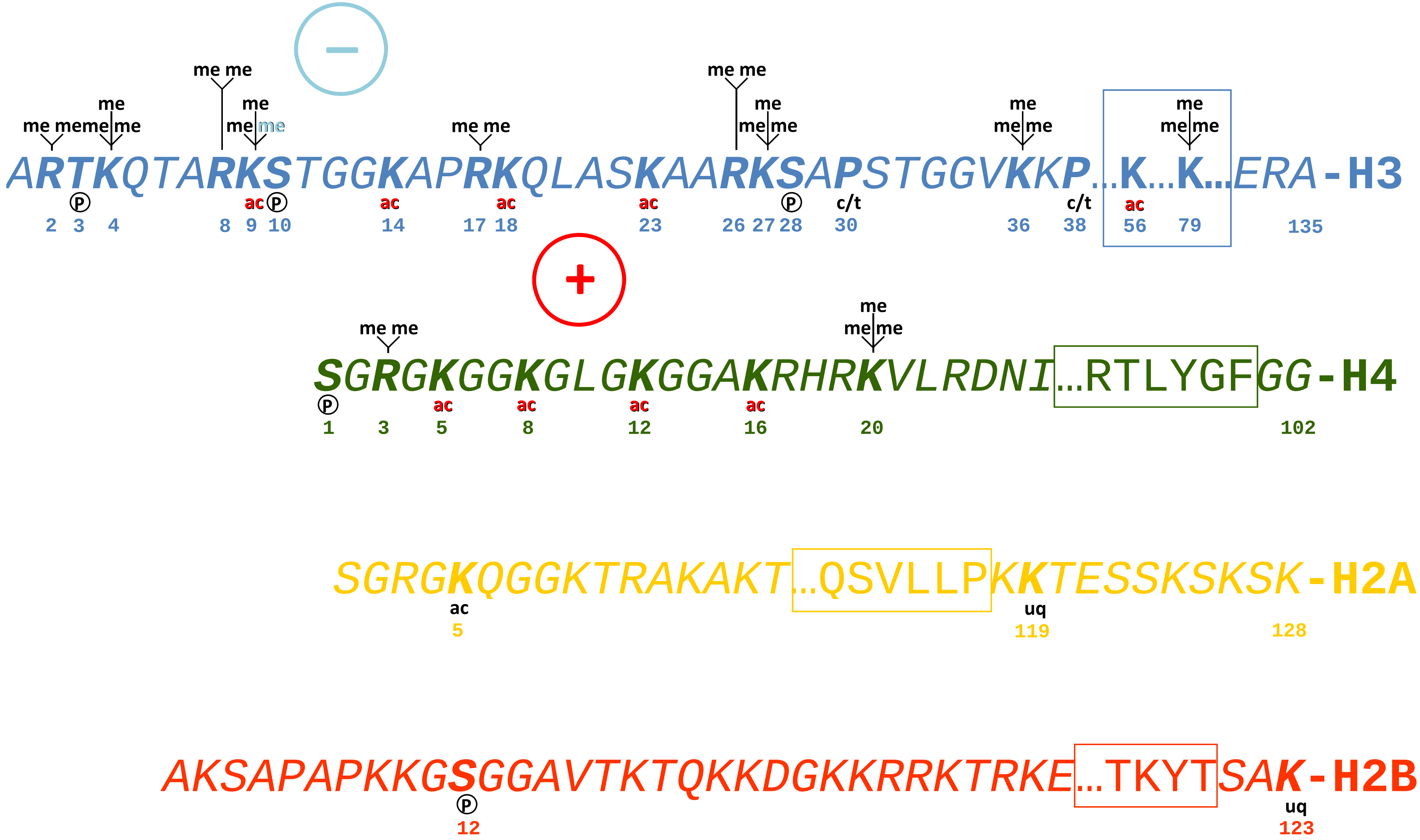
Structure of a nucleosome



- 147 bp DNA wrapped around octamer consisting of dimers of histones **H2A**, **H2B**, **H3** and **H4**

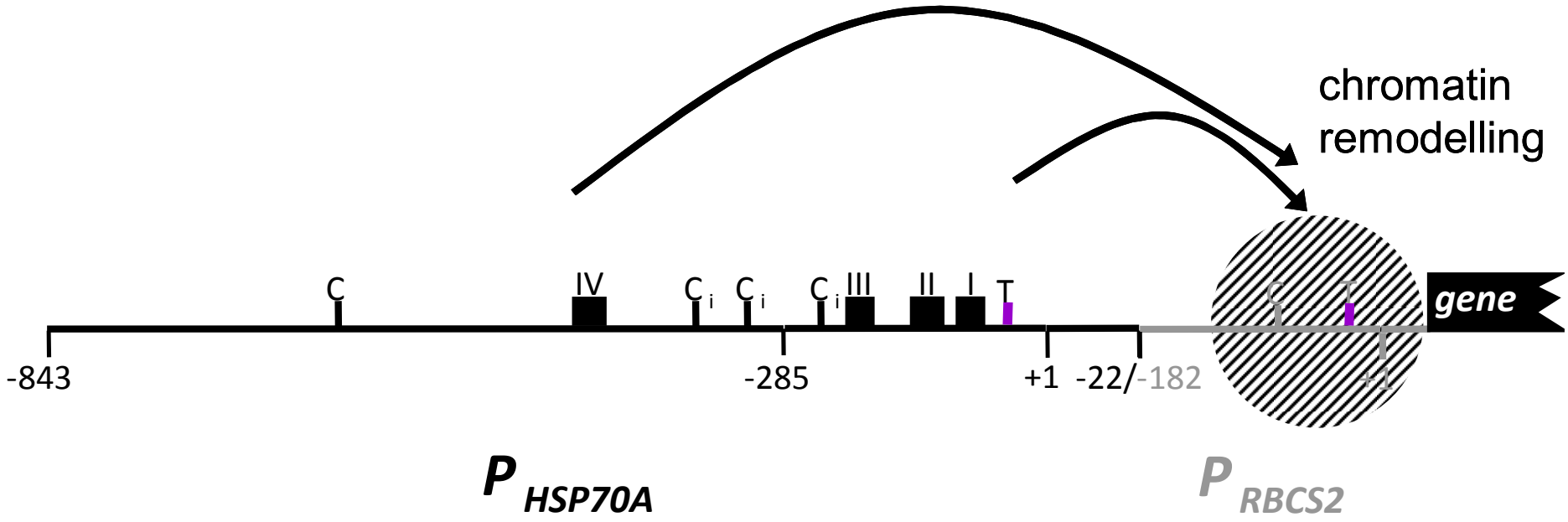
Luger, 2003, Curr. Opin. Genet. Dev., 13, 127-135.

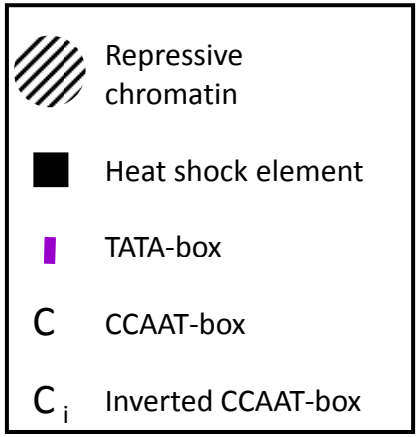
The Histone code



Kouzarides, 2007, Cell, 128, 693-705; Luger et al., 1997, Nature, 389, 251-260.

Model explaining how the *HSP70A* promoter may activate transgenes





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Summary & Outlook

Chloroplast

• Significant transgene expression only in *Chlamydomonas psbA* mutants
→ needs to be established in other algal systems (started in *Euglena gracilis* & *Porphyridium sp*.)

Nucleus

Insertional mutant libraries

• Only established for *Chlamydomonas*
⟶ higher transformation efficiencies needed for other systems

Homologous recombination

• encouraging results with ssDNA in *Chlamydomonas*
• perhaps easy in *C. merolae* and *Chlorella*?!
⟶ Much more research needed

RNA silencing

• Well established for *Chlamydomonas* (antisense, IR, amiRNA)
• Encouraging: first reports on successful IR in *P. tricornutum*; antisense in *C. merolae*
⟶ more research needed; micro-RNAs present in other microalgae?

Transgene expression

• Transient expression appears to work well in *C. merolae*, *Chlorella*, *D. salina* (*N. occulata*?!)
• Expression of stable transgenes appears fine in *P. tricarnutum*, bad in *Chlamydomonas*
⟶ Solvable when epigenetic gene silencing mechanisms understood?