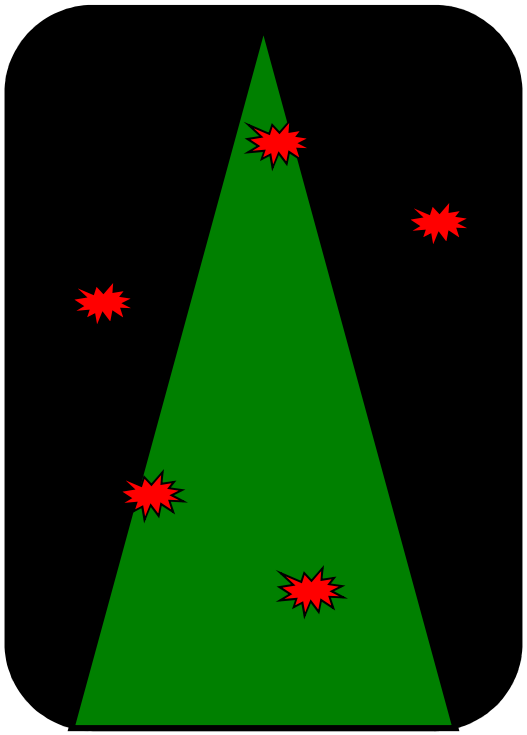
General Concepts to Dissect Signaling Pathways

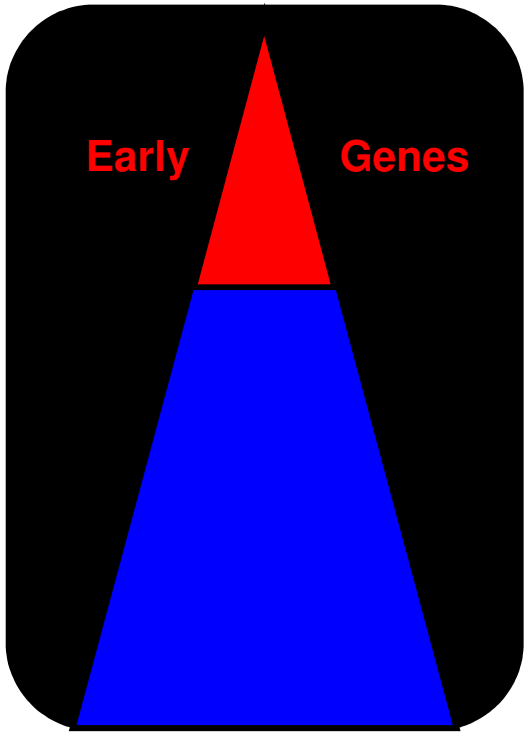
Forward Genetics



Growth & Development

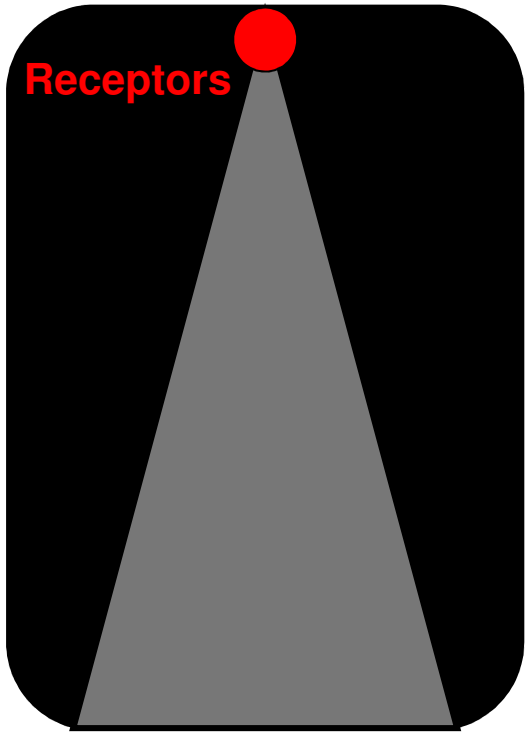
(➔ Biochemistry)

Molecular Biology



Gene Expression

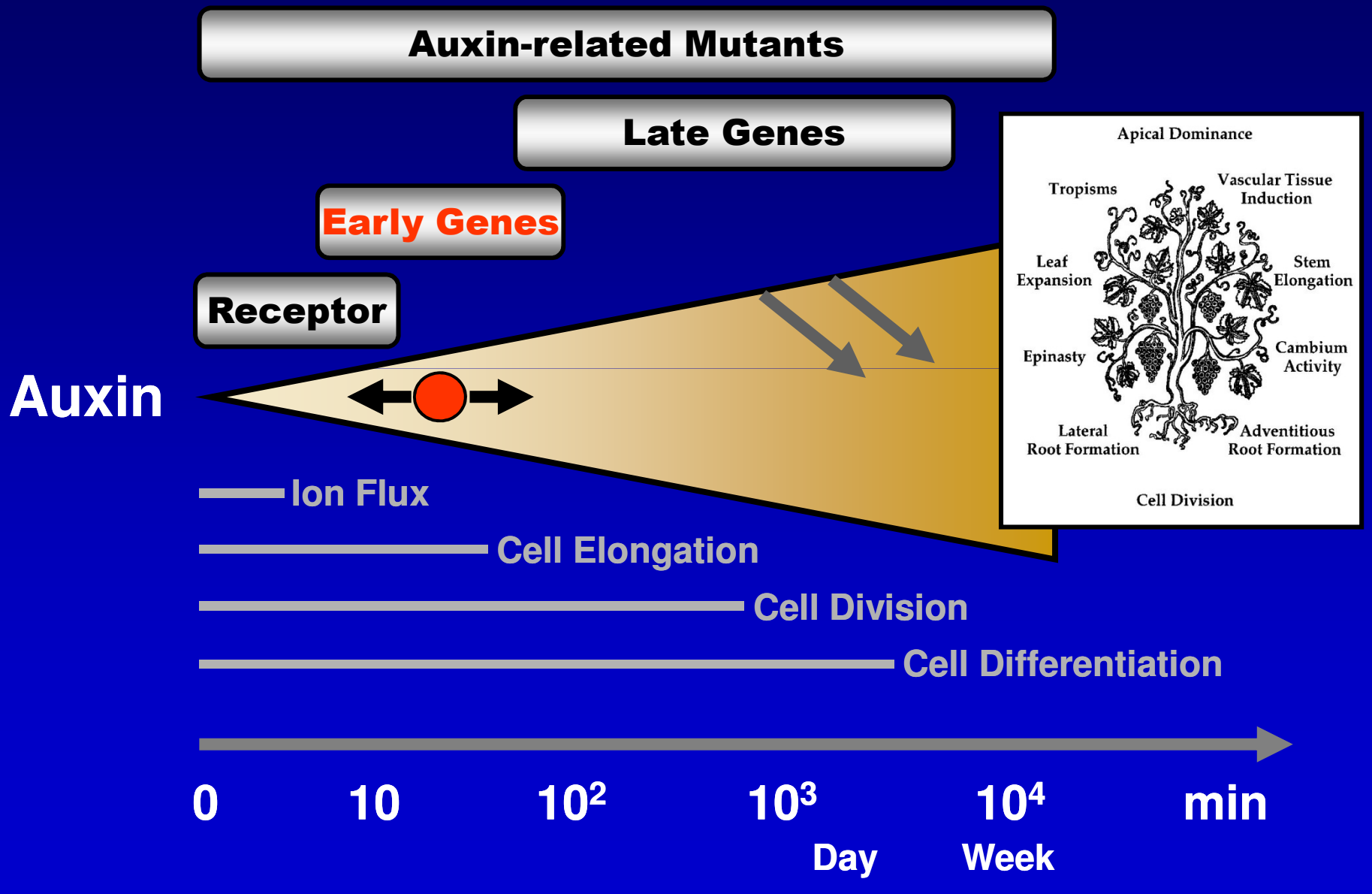
Biochemistry



Binding Proteins

(➔ Reverse Genetics)

Approaches to Dissect Auxin Signaling



Induction of Early (Primary) Response Genes

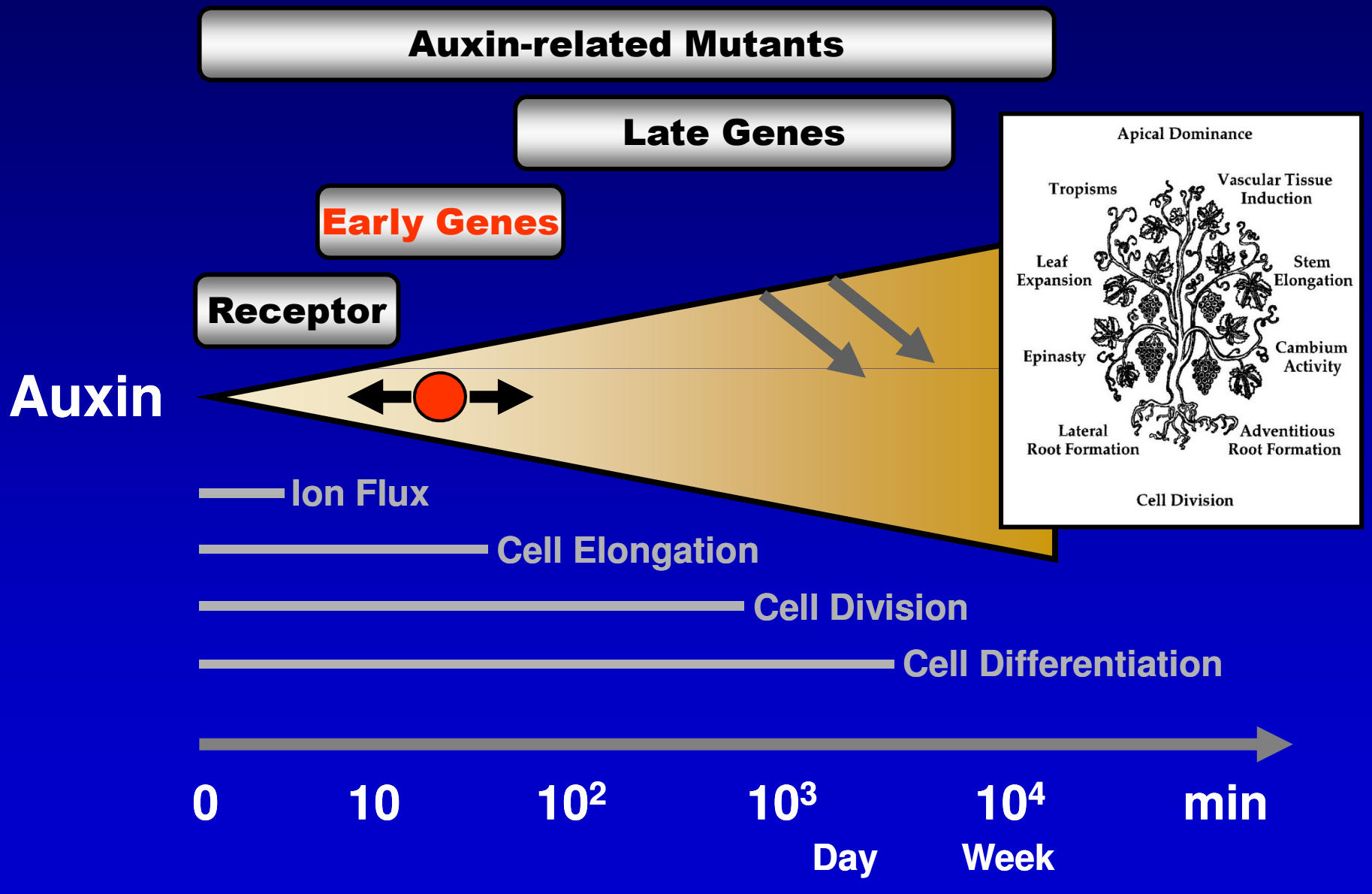
Should be:

- fairly rapid (within a few minutes)
- independent of de novo protein synthesis
- specific for the stimulus

Typically short activation pathway

Typically regulatory functions

Approaches to Dissect Auxin Signaling



Classic Experimental Systems

Straight Growth Assays

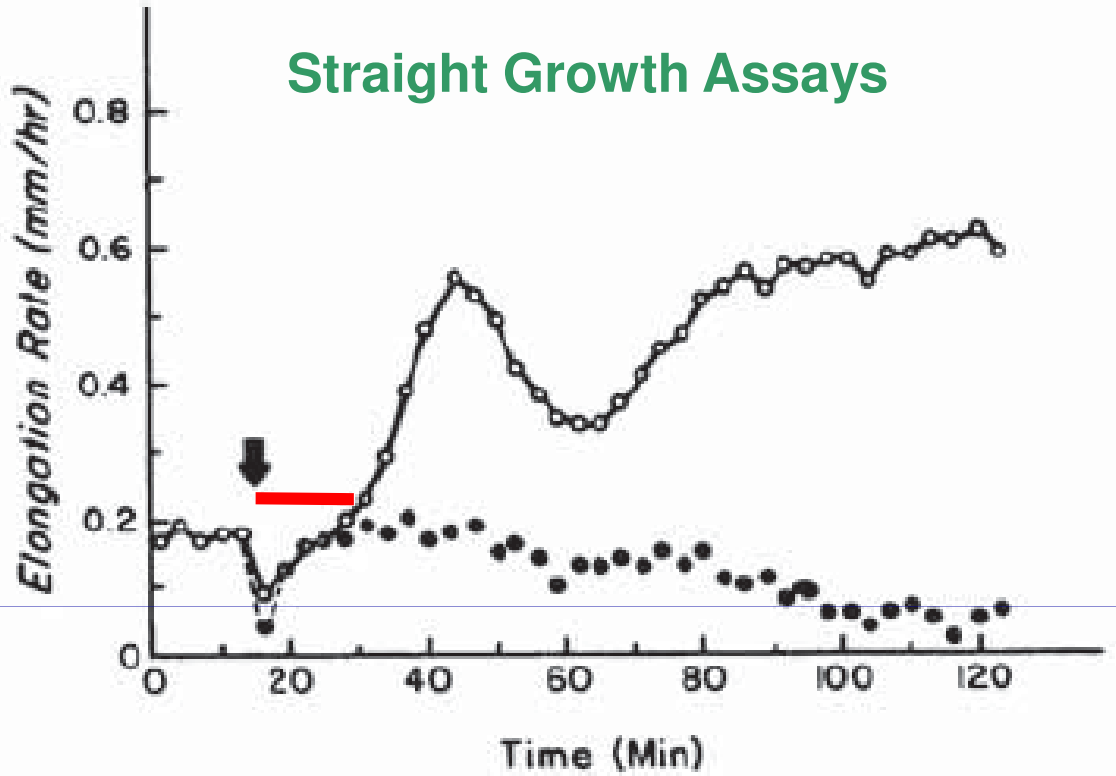


FIG. 1. Effect of auxin on the elongation rate of soybean hypocotyl segments. The segment was preincubated for 60 min in buffered sucrose (5 mM KH_2PO_4, pH 6.0, 30 mM sucrose) before transfer to the growth chamber. After growth was monitored for 15 min, the buffered sucrose was replaced (*arrow*) with an identical solution (●) or buffered sucrose containing 45 μM auxin (○). Rates were determined directly from the growth curve for each 2.5 min interval.

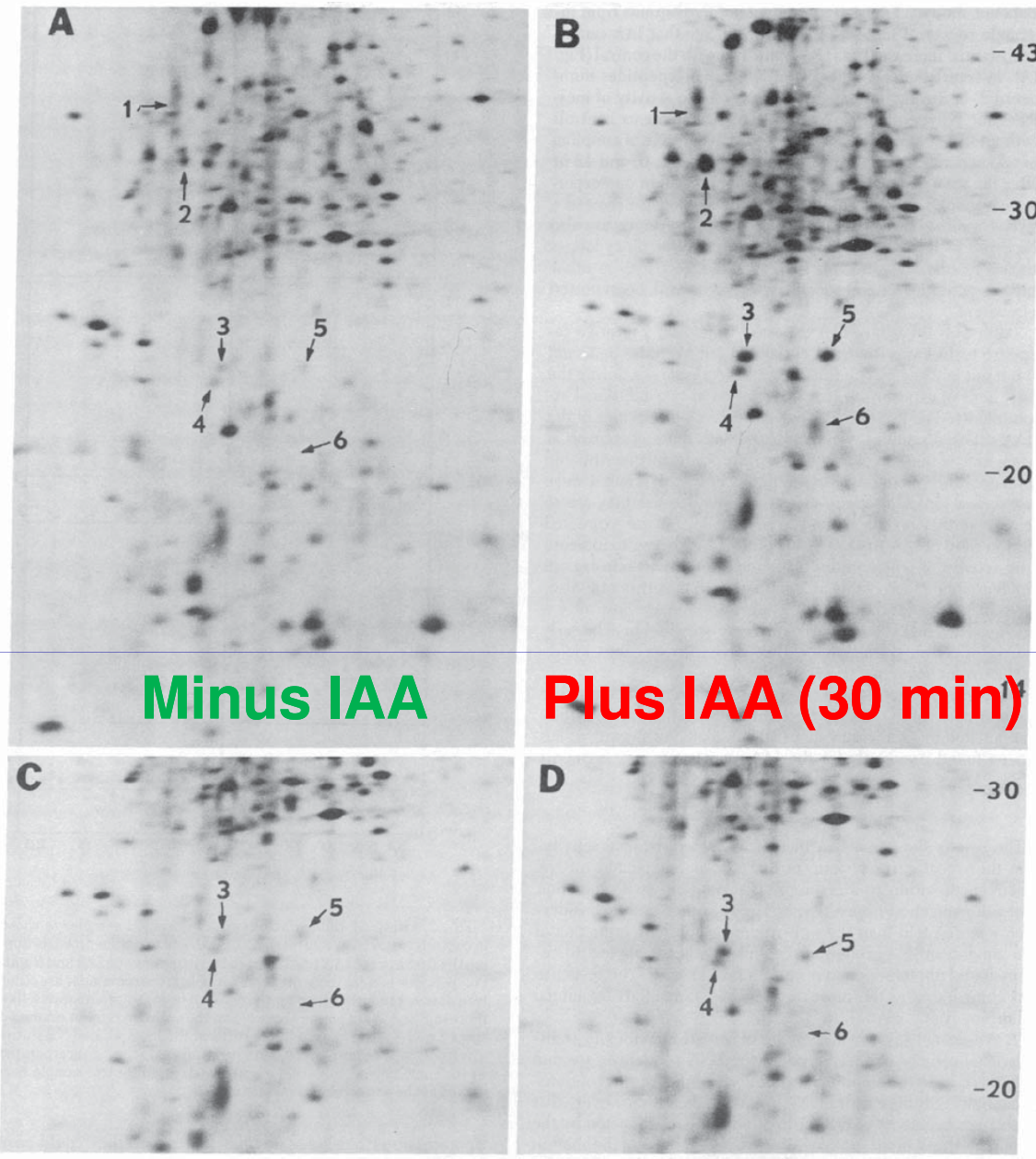
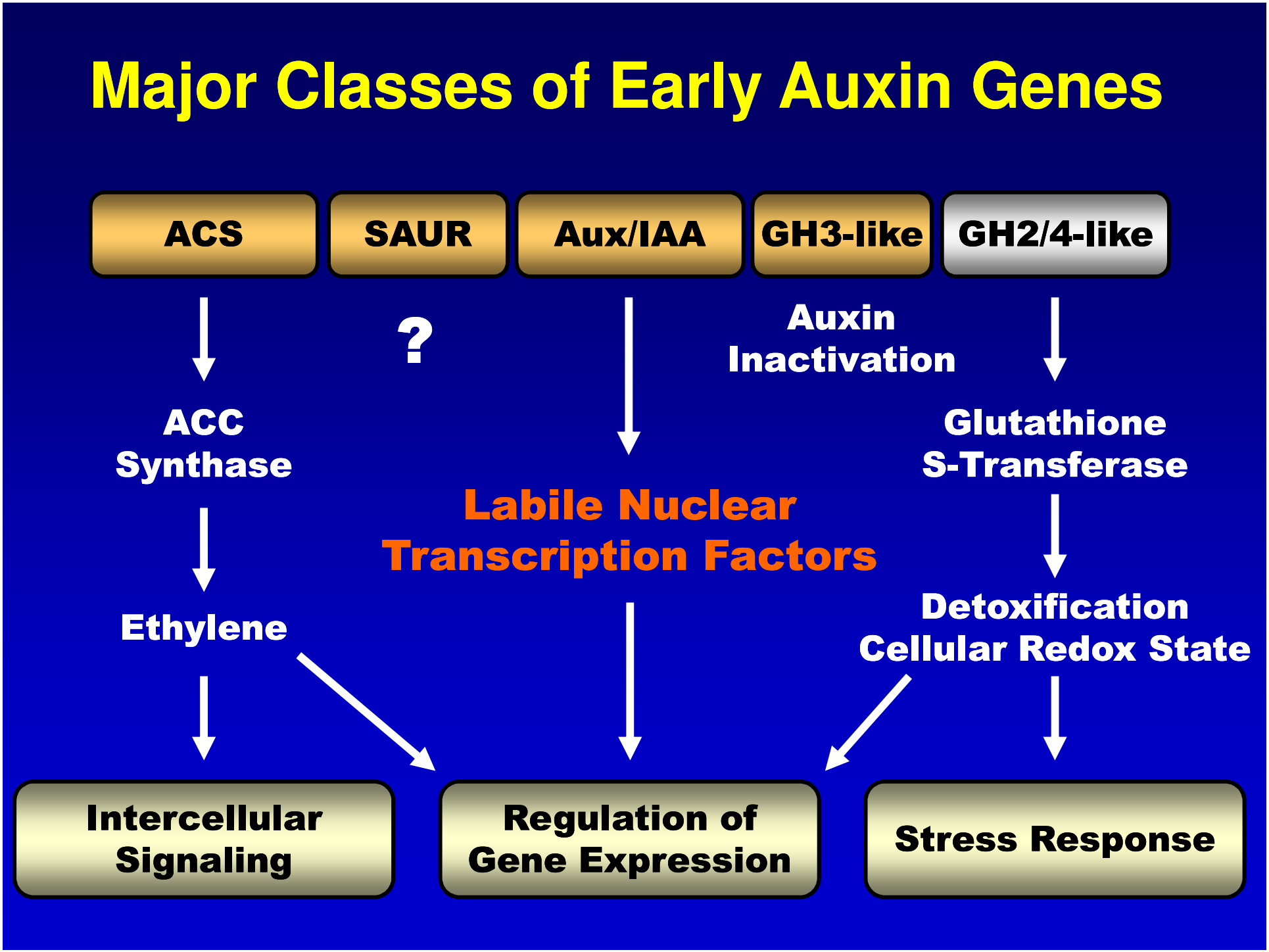
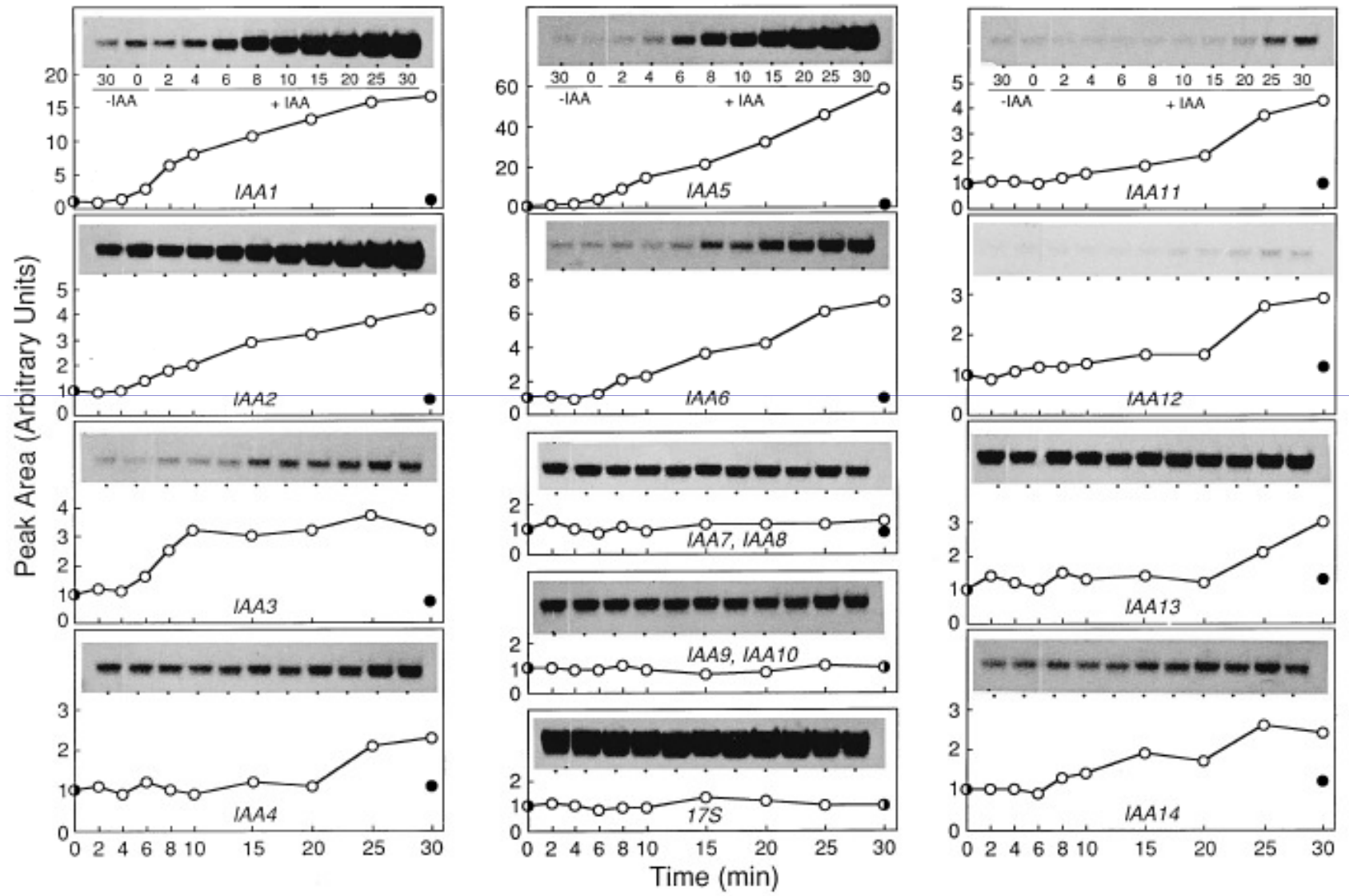


FIG. 1. Portions of autoradiograms of electrophoretically separated [^{35}S]methionine-labeled *in vitro* translation products specified by poly(A)$^+$RNA from pea stem segments. First (horizontal) direction, nonequilibrium pH gradient; second (vertical) direction, NaDodSO$_4$/acrylamide gradient. (*A* and *B*) Translation products with a M_r range of 14,000–50,000 and a pH range from 4.0 (left) to 8.0 (right). (*C* and *D*) Translation products with M_rs of 18,000–30,000 from comparable electropherograms. Poly(A)$^+$mRNA was from segments kept 2 hr without auxin after cutting (*C*) and from segments treated as in *C* but with either an additional 30-min incubation with 20 μM IAA (*D*) or an additional 2-hr incubation without (*A*) and with (*B*) 20 μM IAA. M_rs of reference proteins are shown on the right $\times 10^{-3}$: α-lactalbumin, 14; soybean trypsin inhibitor, 20; carbonic anhydrase, 30; ovalbumin, 43.

Major Classes of Early Auxin Genes



Some Features of Aux/IAA Genes: Induction Kinetics



Short-term kinetics of mRNA accumulation in response to IAA. See the legend to Figure 5.

Some Features of Aux/IAA Genes: Specificity

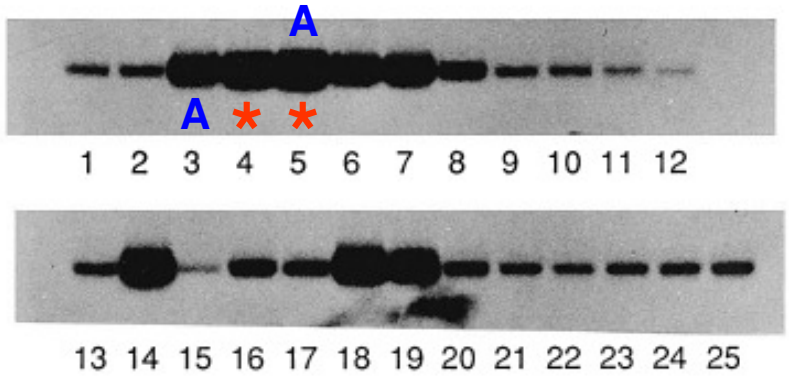
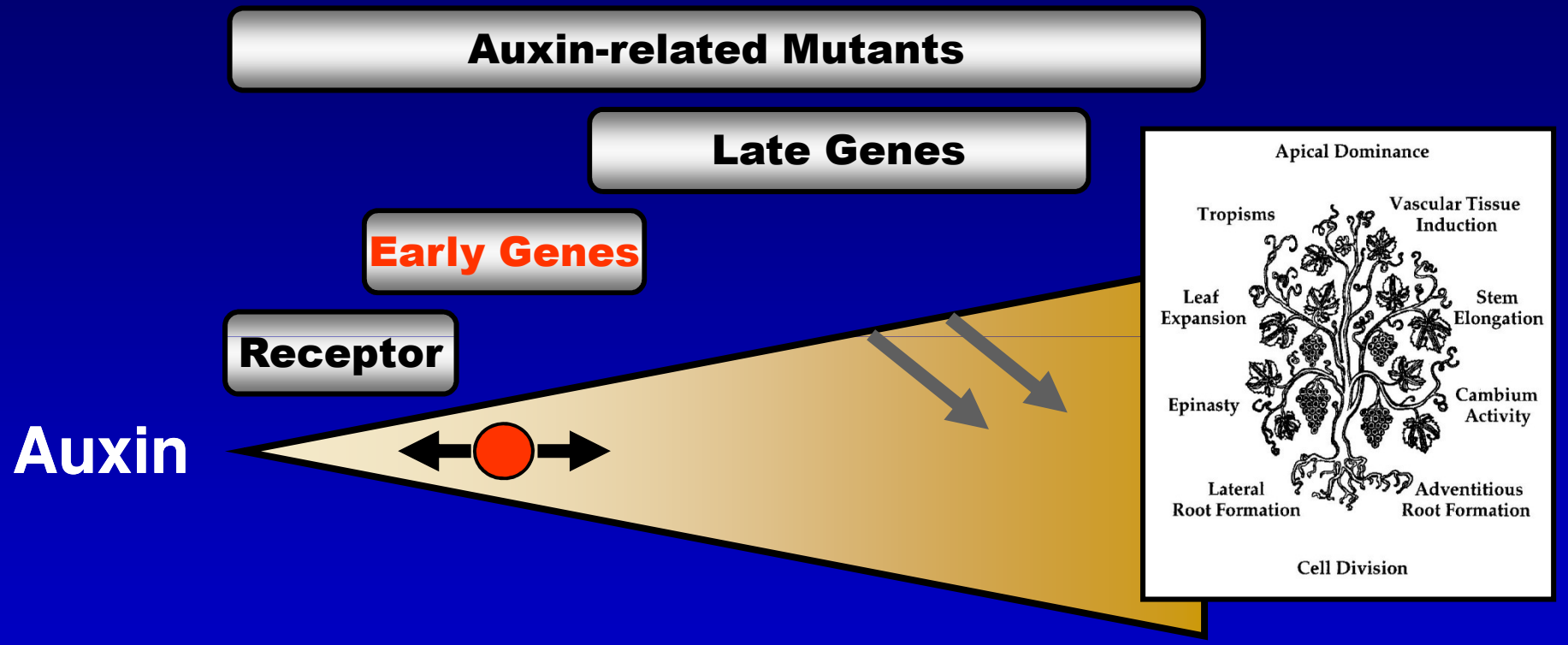


Figure 4. Specificity of the hormonal response. Total RNA (25 μg) from six days old etiolated seedlings treated for one hour with various chemicals and conditions, if not otherwise indicated, were hybridized with a ^{32}P-labeled *IAA1*-specific probe. The lanes are: 1, untreated; 2 and 13, control-treated; 3 and 14, 20 μM IAA; 4, 50 μM CHX; 5, 20 μM IAA and 50 μM CHX after 30 minutes pretreatment with 50 μM CHX only; 6, 20 μM 2,4-D; 7, 20 μM α-NAA; 8, 20 μM PAA; 9, 20 μM L-tryptophan; 10, wounding; 11, 0.5 M sorbitol; 12, heat treatment at 42°C for 15 minutes followed by 45 minutes recovery at room temperature; 15, 20 μM ABA; 16, 20 μM GA; 17, 20 μM BA; 18, 20 μM IAA and 20 μM BA; 19, 20 μM IAA/20 μM BA and 50 mM LiCl; 20, 50 mM LiCl; 21, 10 ppm ethylene; 22, N_2; 23, air control; 24, control (six hours in 10 mM phosphate); 25, phosphate starvation (six hours, no phosphate present).

Effect of CHX *

Approaches to Dissect Auxin Signaling



Promoter ←● → **Protein?**

Aux/IAA Genes

Domain Structure of Aux/IAA Proteins

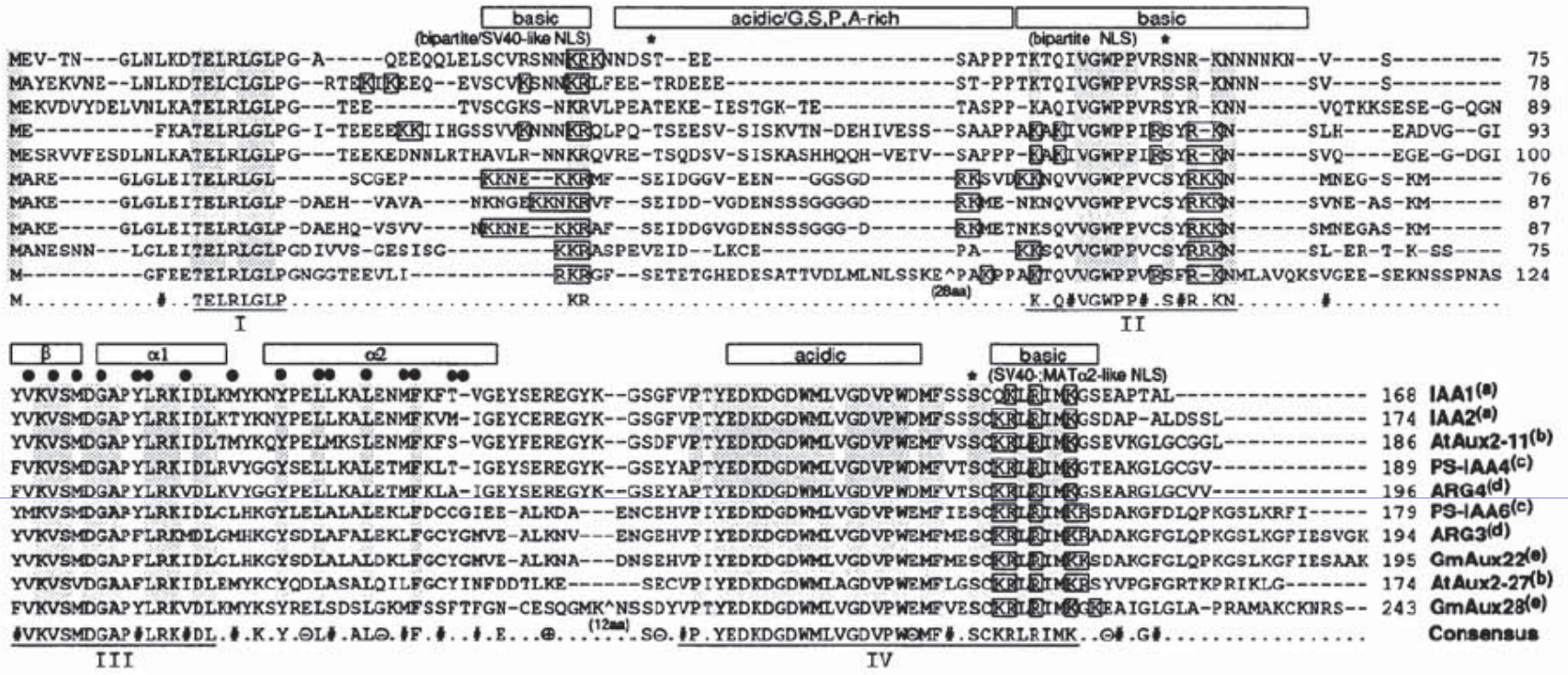


FIG. 1. Sequence alignment and domain structure of primary auxin-responsive gene products. Identical (shaded) and generally conserved amino acid residues appear in the consensus {at least 8 of 10 matches [⊖, acidic (D, E); ⊕, basic (R, K); #, hydrophobic (A, C, V, I, L, M, F, Y, W)]}. Conserved domains are underlined and indicated by Roman numerals. Basic residues that may contribute to putative nuclear localization signals (NLS) are boxed (26). Conserved phosphorylation sites proximal to putative NLS are indicated by stars on top of the alignment (casein kinase II protein kinase, S/TXXE/D; protein kinase C, S/TXR/K). Amino acids that may form hydrophobic surfaces in the predicted conserved amphipathic $\beta\alpha\alpha$ motif are indicated by ●. Sources of the sequences are as follows: a and b, *Arabidopsis thaliana* (S.A. and A.T., unpublished data and ref. 11); c, pea (*Pisum sativum*) (12); d, mung bean (*Vigna radiata*) (10); e, soybean (*Glycine max*) (9).

No obvious similarity to any other proteins! What to do??

Question:
Where are the proteins expressed *in planta*?

First Approach:
Immunolocalization → BIG FAILURE !!!

Proc. Natl. Acad. Sci. USA
Vol. 91, pp. 326–330, January 1994
Biochemistry

Early auxin-induced genes encode short-lived nuclear proteins

(plant hormone action/plant cell growth/protein stability/$\beta\alpha\alpha$ DNA binding motif/nuclear localization)

STEFFEN ABEL, PAUL W. OELLER*, AND ATHANASIOS THEOLOGIS†

Plant Gene Expression Center, 800 Buchanan Street, Albany, CA 94710

Communicated by Kenneth V. Thimann, October 4, 1993 (received for review August 11, 1993)

... because Aux/IAA proteins are labile

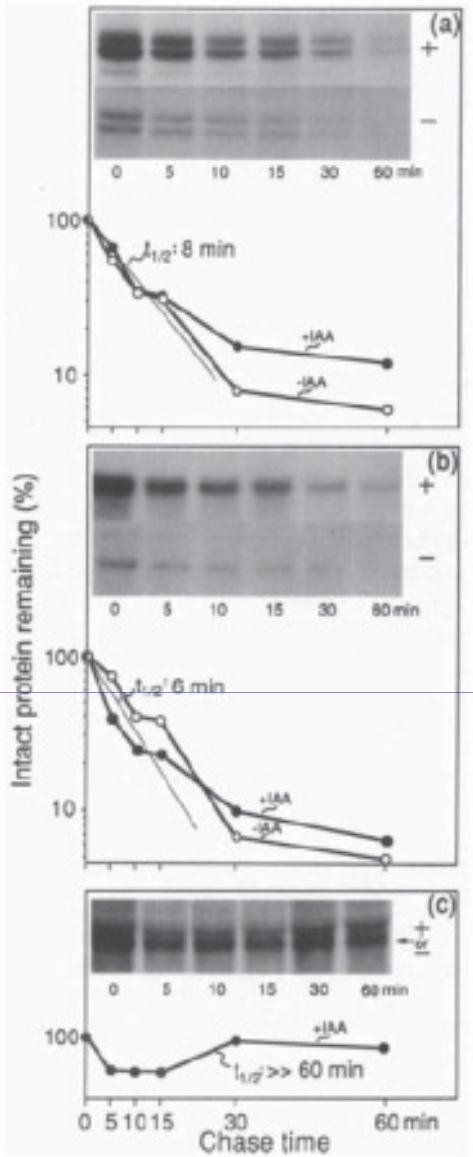


FIG. 2. $t_{1/2}$ of PS-IAA4 and PS-IAA6 proteins. Etiolated pea epicotyl tissue was pulse-labeled *in vivo* with [^{35}S]methionine in the presence (+) or absence (−) of IAA for 2 hr, chased in the presence of excess unlabeled methionine for the times indicated, and processed for immunoprecipitation using affinity-purified PS-IAA4/5 (*a*), PS-IAA6 (*b*), and β-tubulin (*c*) antibodies. Portions of the fluorograms are shown. The results were quantitified with a scanning densitometer and presented graphically.

... but where are the proteins?

Hypothesis: In the cell nucleus.
(basic motifs similar to nulear localization signals)

FIG. 1. Sequence alignment and domain structure of primary auxin-responsive gene products. Identical (shaded) and generally conserved amino acid residues appear in the consensus {at least 8 of 10 matches [⊖, acidic (D, E); ⊕, basic (R, K); #, hydrophobic (A, C, V, I, L, M, F, Y, W)]}. Conserved domains are underlined and indicated by Roman numerals. Basic residues that may contribute to putative nuclear localization signals (NLS) are boxed (26). Conserved phosphorylation sites proximal to putative NLS are indicated by stars on top of the alignment (casein kinase II protein kinase, S/TXXE/D; protein kinase C, S/TXR/K). Amino acids that may form hydrophobic surfaces in the predicted conserved amphipathic $\beta\alpha\alpha$ motif are indicated by ●. Sources of the sequences are as follows: a and b, *Arabidopsis thaliana* (S.A. and A.T., unpublished data and ref. 11); c, pea (*Pisum sativum*) (12); d, mung bean (*Vigna radiata*) (10); e, soybean (*Glycine max*) (9).

The data say: Yes, they are!

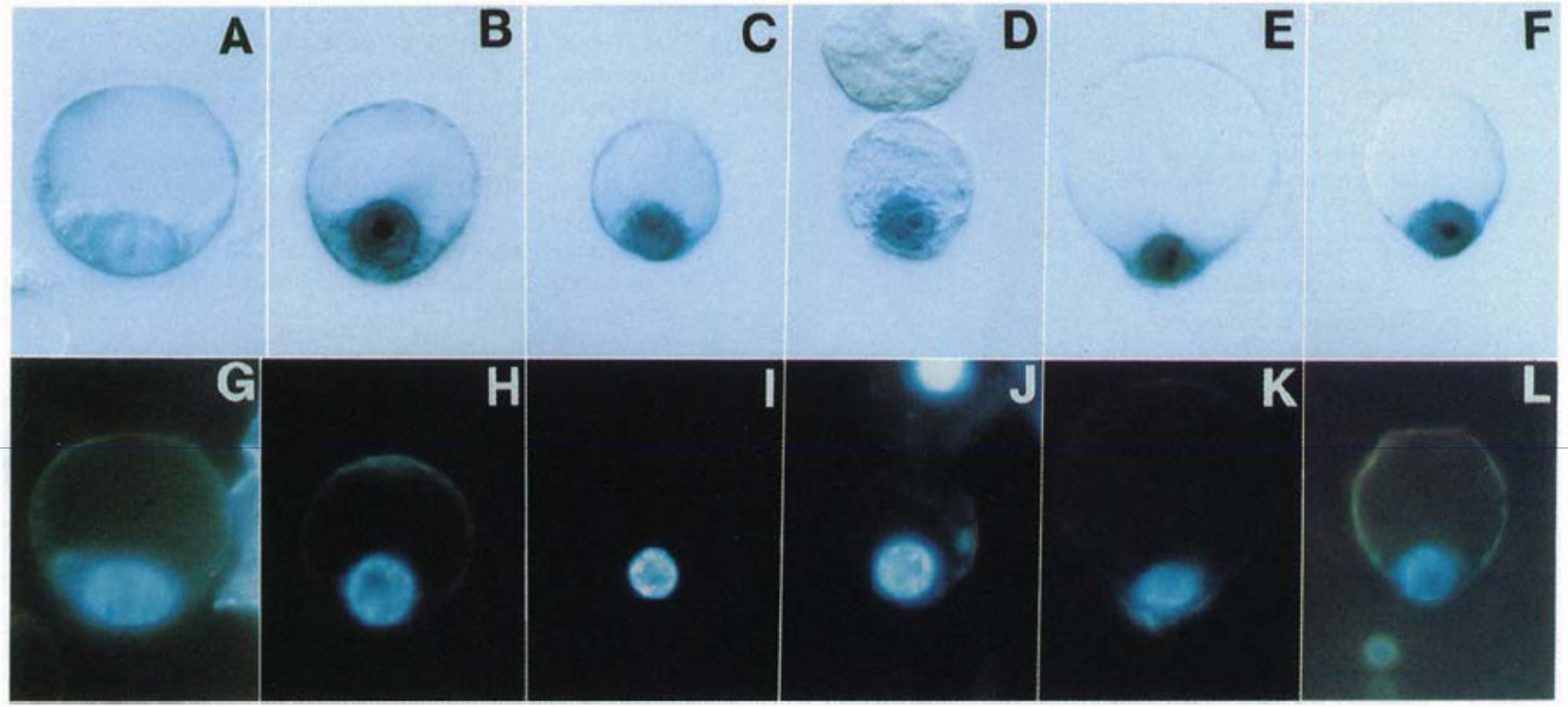


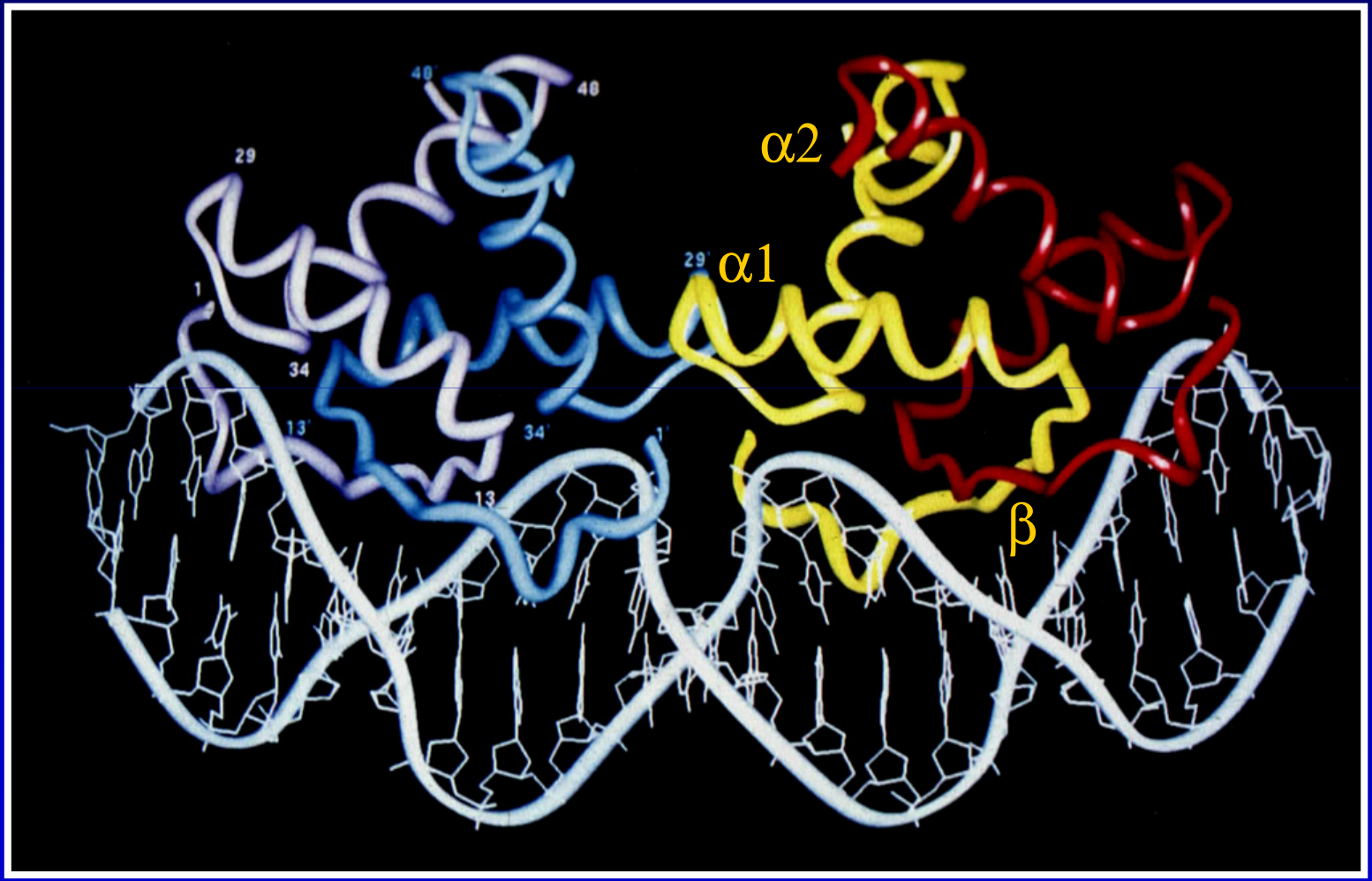
FIG. 3. Nuclear localization of the auxin-regulated polypeptides. Tobacco (*Nicotiana tabacum*) protoplasts were purified, transfected with plasmid DNA (containing *GUS*–auxin gene fusions), incubated in culture medium for 12–16 hr, and assayed for GUS activity (*A–F*) and stained for nuclei (*G–L*). (*A* and *G*) Authentic GUS. (*B* and *H*) GUS::VirD2. (*C* and *I*) GUS::PS-IAA4. (*D* and *J*) GUS::PS-IAA6. (*E* and *K*) GUS::IAA1. (*F* and *L*) GUS::IAA2.

Translational fusion constructs
(transient expression in tobacco protoplasts)

Domain III is similar to the DNA-binding domain of prokaryotic transcription factors

FIG. 1. Sequence alignment and domain structure of primary auxin-responsive gene products. Identical (shaded) and generally conserved amino acid residues appear in the consensus {at least 8 of 10 matches [⊖, acidic (D, E); ⊕, basic (R, K); #, hydrophobic (A, C, V, I, L, M, F, Y, W)]}. Conserved domains are underlined and indicated by Roman numerals. Basic residues that may contribute to putative nuclear localization signals (NLS) are boxed (26). Conserved phosphorylation sites proximal to putative NLS are indicated by stars on top of the alignment (casein kinase II protein kinase, S/TXXE/D; protein kinase C, S/TXR/K). Amino acids that may form hydrophobic surfaces in the predicted conserved amphipathic $\beta\alpha\alpha$ motif are indicated by ●. Sources of the sequences are as follows: a and b, *Arabidopsis thaliana* (S.A. and A.T., unpublished data and ref. 11); c, pea (*Pisum sativum*) (12); d, mung bean (*Vigna radiata*) (10); e, soybean (*Glycine max*) (9).

Co-Crystal Structure of Arc



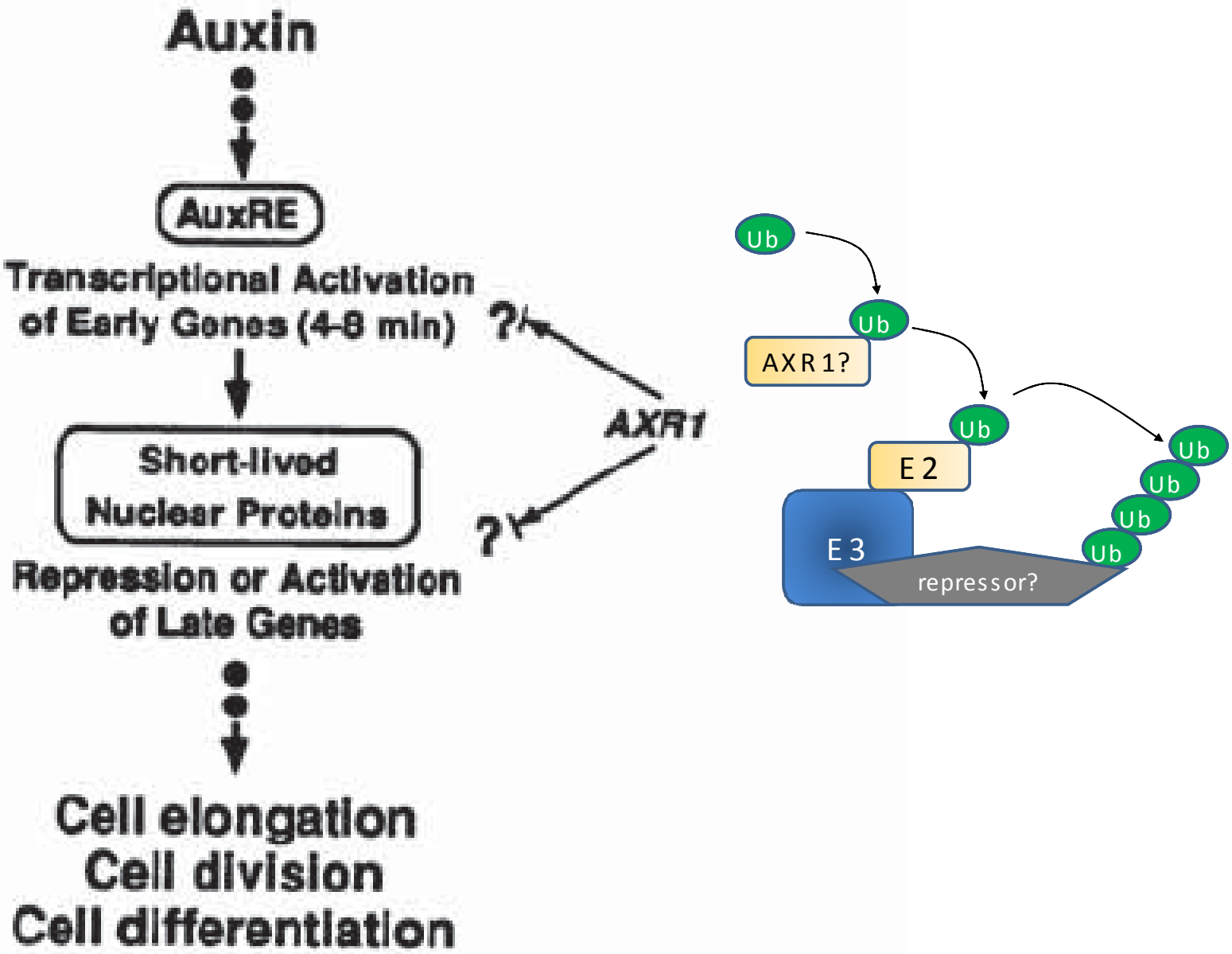


FIG. 5. Model for early auxin events.

Approaches to Dissect Auxin Signaling

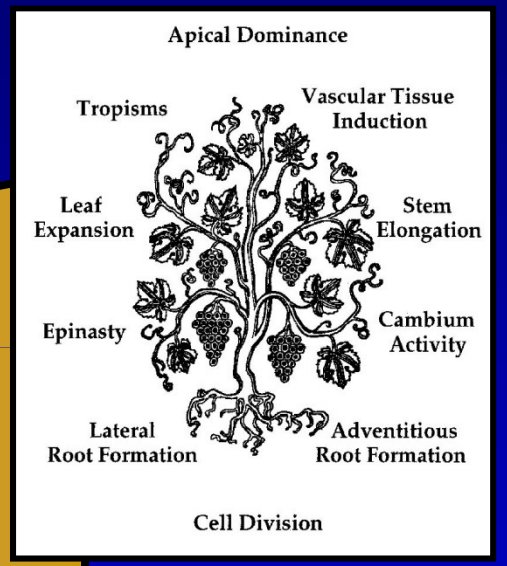
Auxin-related Mutants

Late Genes

Early Genes

Receptor

Auxin



Promoter (AuxRE) ?

Proteins

J. Mol. Biol. (1993) 233, 580–596

Identification of the Auxin-responsive Element, *AuxRE*, in the Primary indoleacetic Acid-inducible Gene, *PS-IAA4/5*, of Pea (*Pisum sativum*)

Nurit Ballas†, Lu-Min Wong† and Athanasios Theologis‡

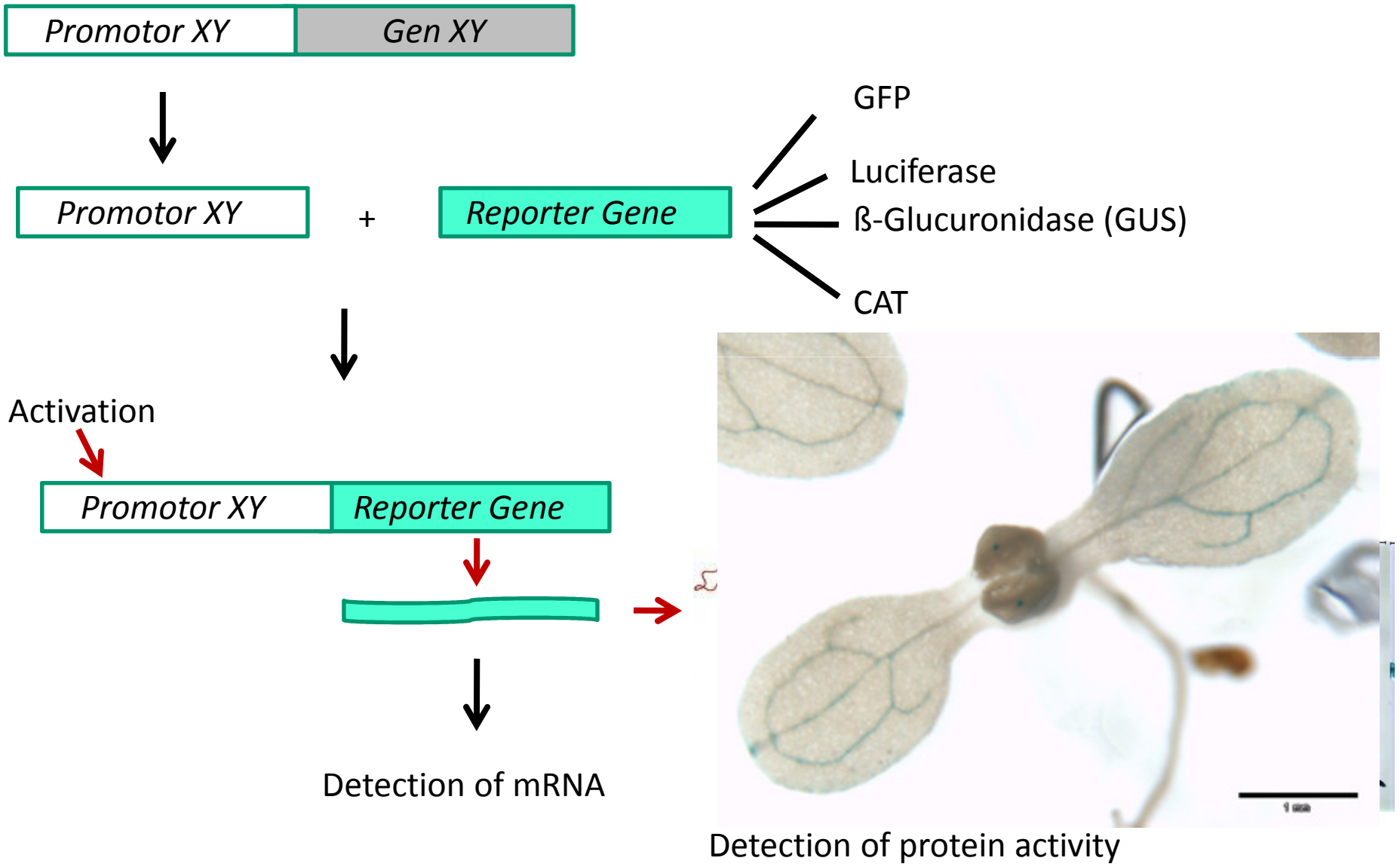
Goal:

Identification of promoter sequences mediating auxin-dependent transcritptional activation of early auxin response genes **(„promoter bashing“)**

Auxin Response Element (AuxRE)

AuxRE → Transcription factors → → → Receptor

Transcriptional Fusions (chimeric plasmid constructs)



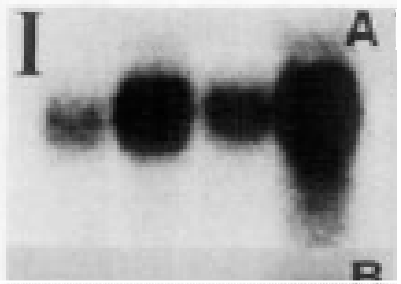
Voraussetzung:

- Etablierung eines Auxin-induzierbaren Protoplastensystems / Reportersystems
  - Lokalisation des AuxRE erfordert das Testen vieler verschiedener (chimärer) Konstrukte
  - stabile Transformation (sehr aufwändig + zeitintensiv + funktioniert nicht mit jeder Pflanzenart gleich gut)
  - ➢ transiente Transfektion mit Protoplasten (leichter zugänglich für die Aufnahme von DNA-Konstrukten und Chemikalien als komplexe Gewebe)

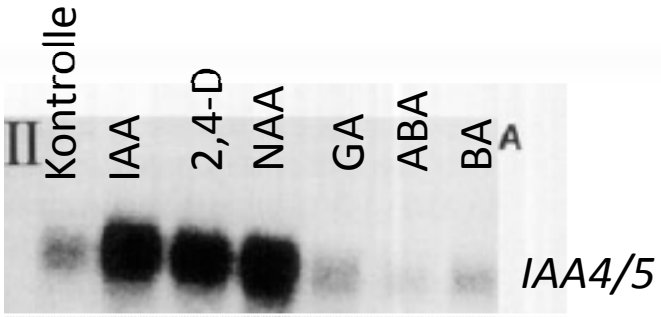
- Notwendiger Test:

  Überprüfen ob sich die Protoplasten genauso verhalten wie intakte Keimlinge (Auxin response)

test protoplasts vs. epicotyls



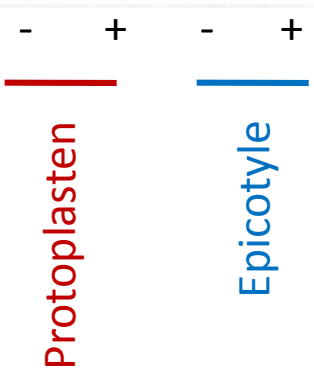
IAA4/5

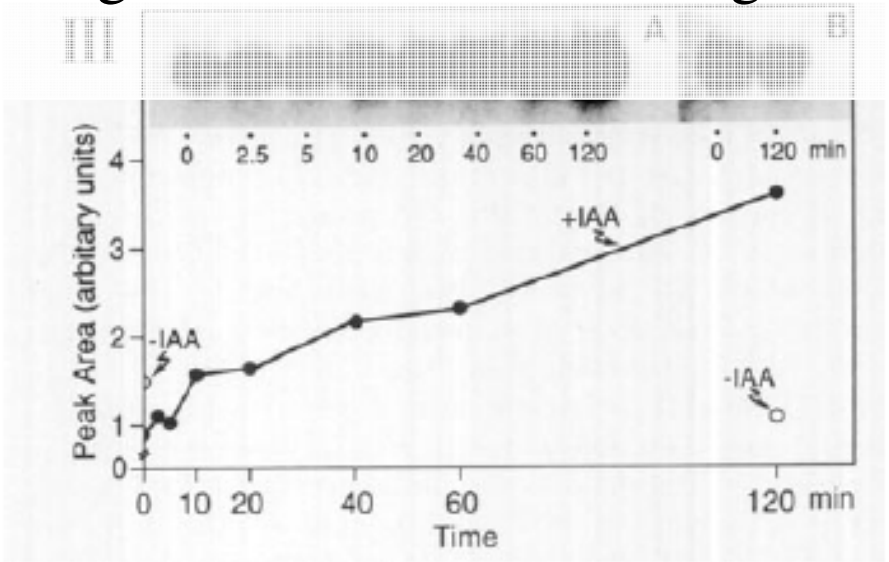


IAA4/5

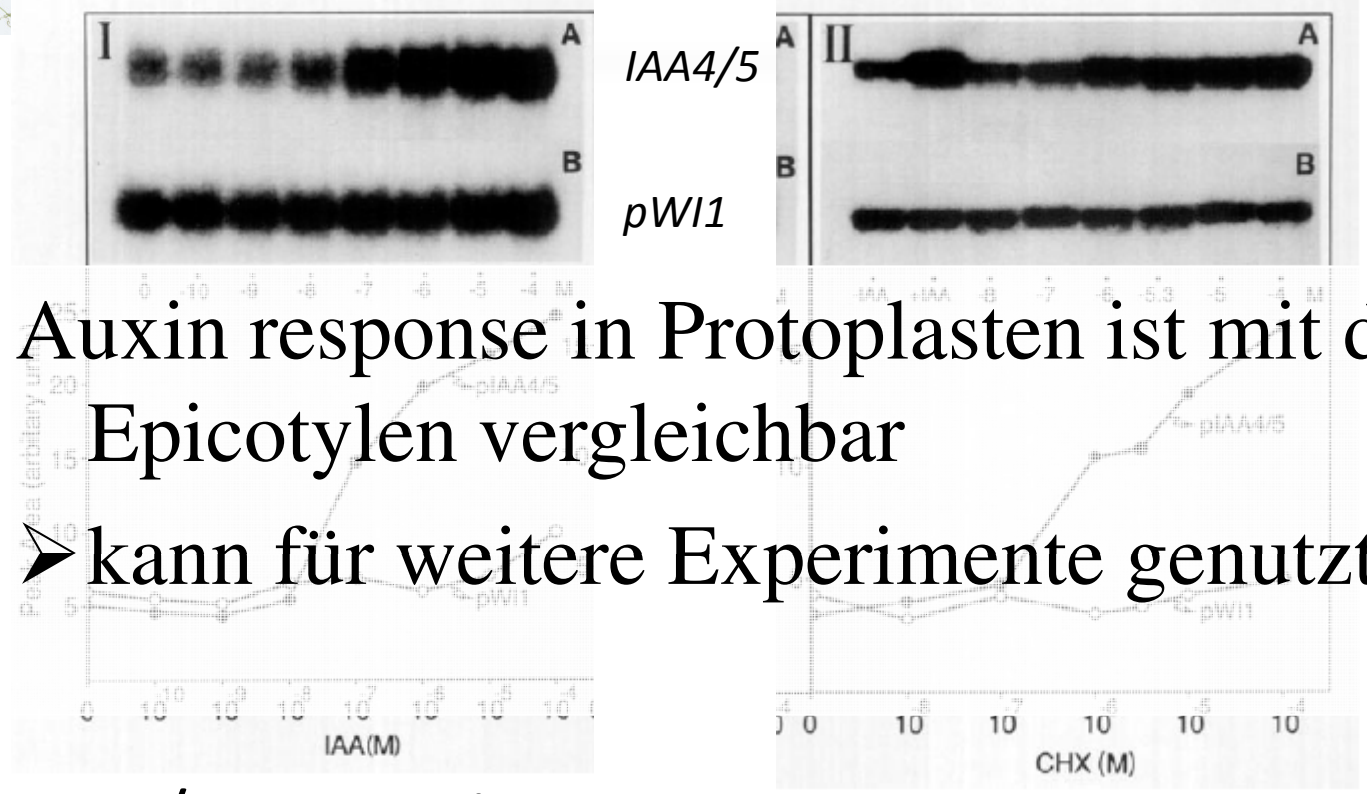
IAA4/5 Induktion in Protoplasten

- gleicht der in Epicotylen
- wird spezifisch durch Auxine induziert
- erfolgt schnell, Transkriptmenge akkumuliert in Abhäng. der Behandlungsdauer





Konz. abhängigkeit



Auxin response in Protoplasten ist mit der von Epicotylen vergleichbar

➢ kann für weitere Experimente genutzt werden

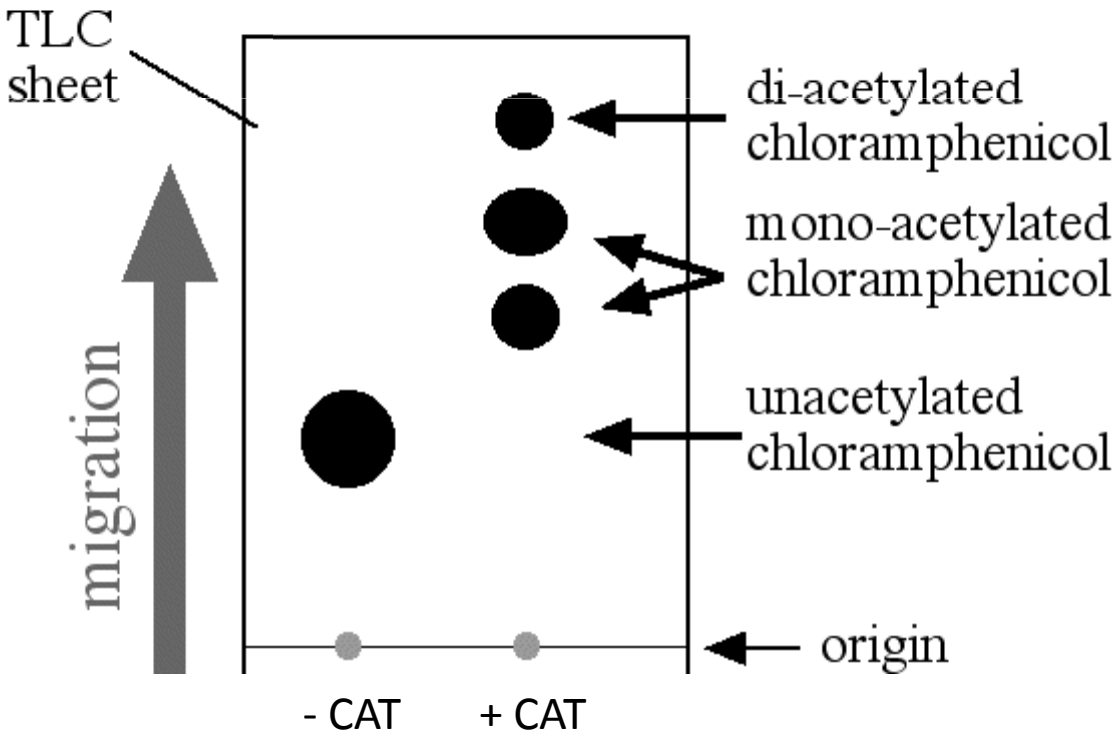
IAA4/5 Expression

- nimmt mit steigener Auxinkonzentration zu
- Inhibierung der Proteinbiosynthese aktiviert Expression auch in Abwesenheit von Auxin!

1. Signalelemente müssen nicht erst gebildet werden
2. Aktivierung im Grundzustand vermutlich durch **labile Repressorproteine?**

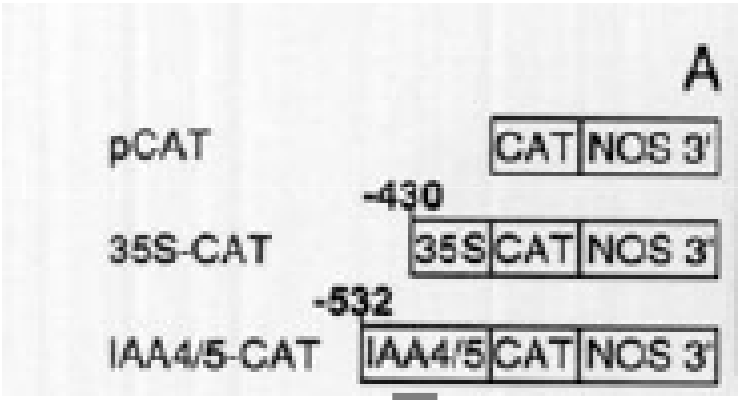
CAT reporter system

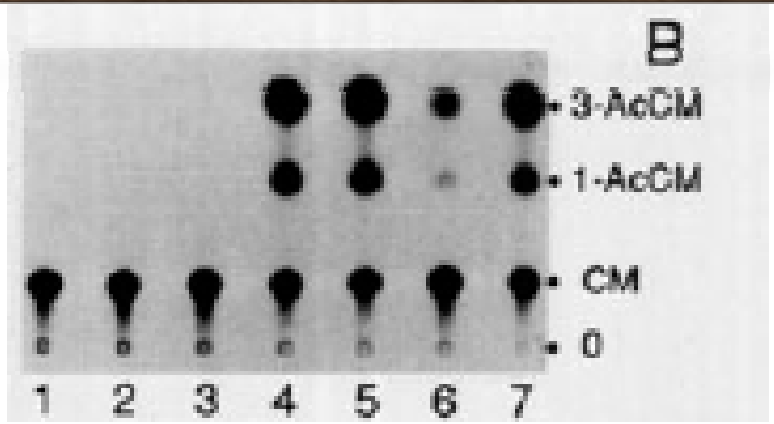
CAT = Chloramphenicol acetyltransferase
Enzym kann das Antibiotikum Chloramphenicol inaktivieren, indem Acetylgruppen übertragen werden



CAT reporter system

Test des CAT reporter systems



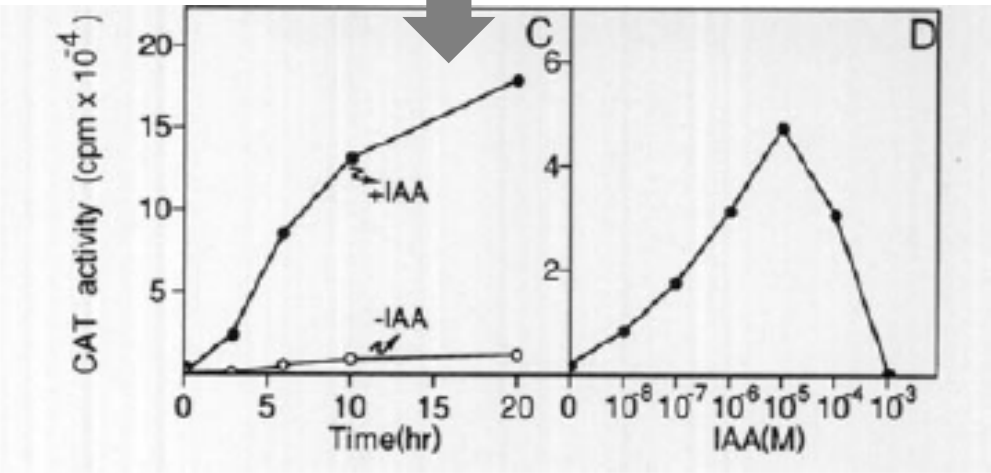


K

kein Promotor

konstitutiver Promotor

IAA4/5 Promotor



IAA4/5::CAT

CAT-Aktivität spiegelt die Auxin response des Promotors zeit- und konzentrationsabhängig wider

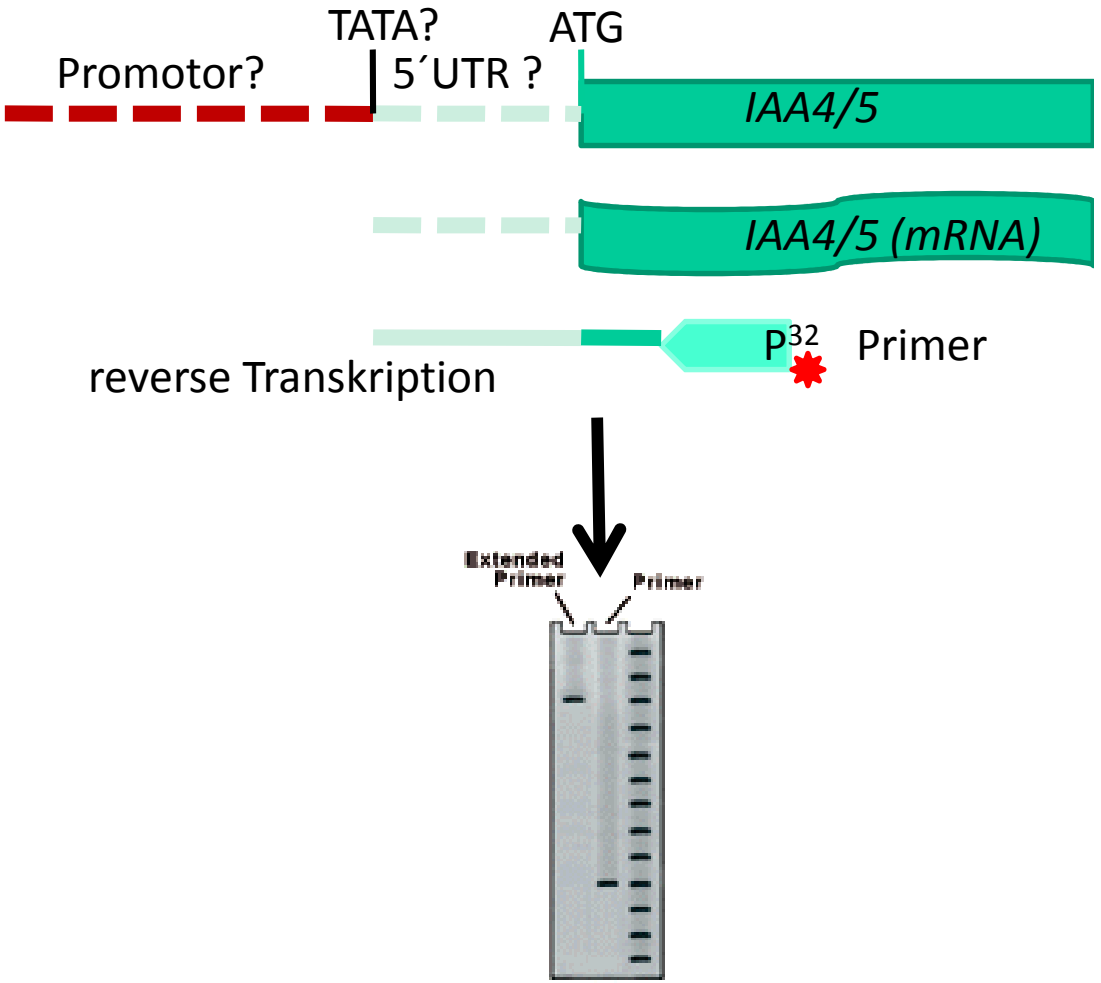
Aufklärung der Promotorstruktur

primer extension:

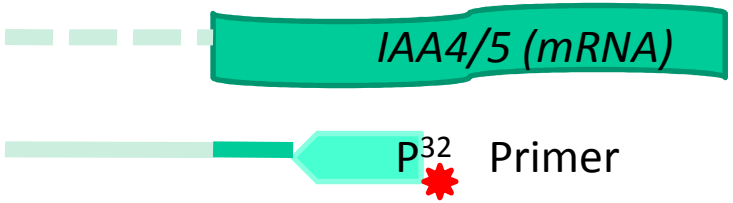
- i.d.R. erster Schritt zur Charakterisierung von Promotorbereichen
- Erhalt vollständiger Transkripte

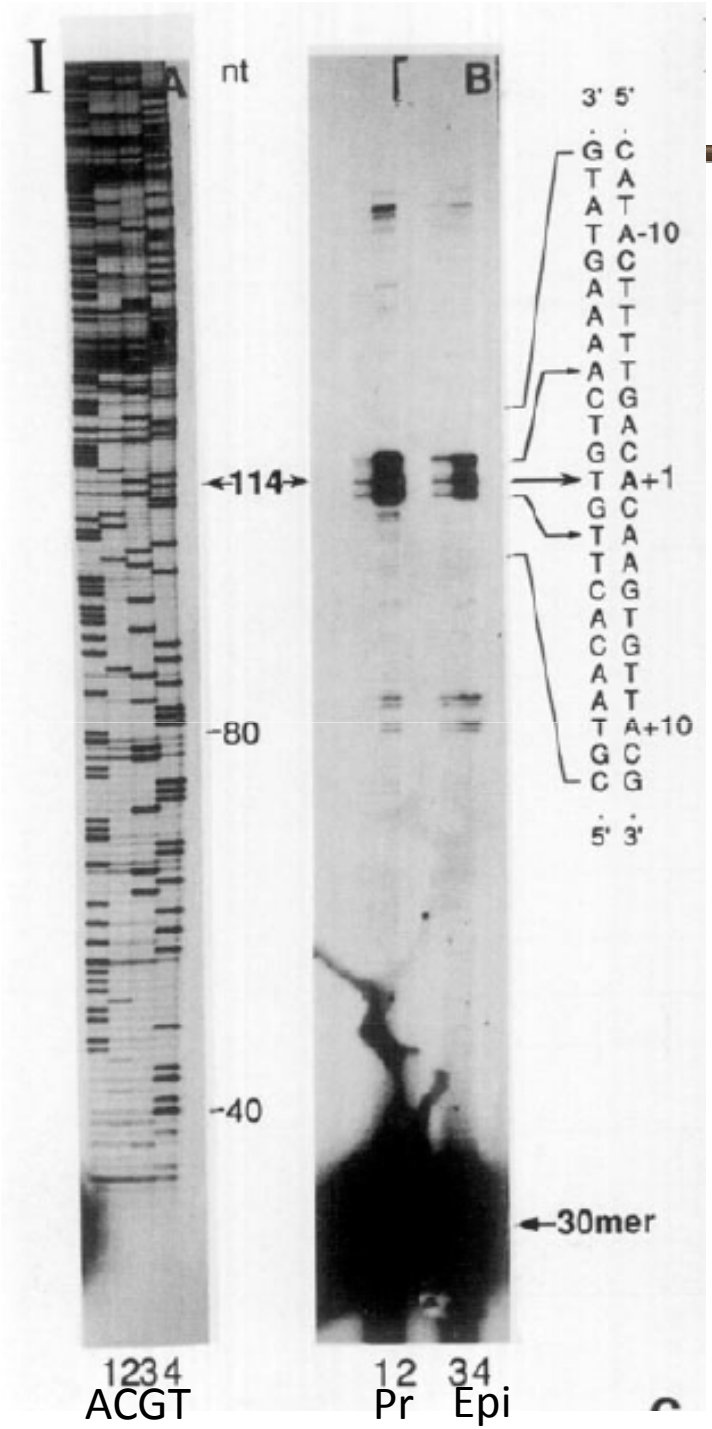
➢ Identifizierung des Transkriptionsstarts

(TATA box, 5´UTR)



Primer extension

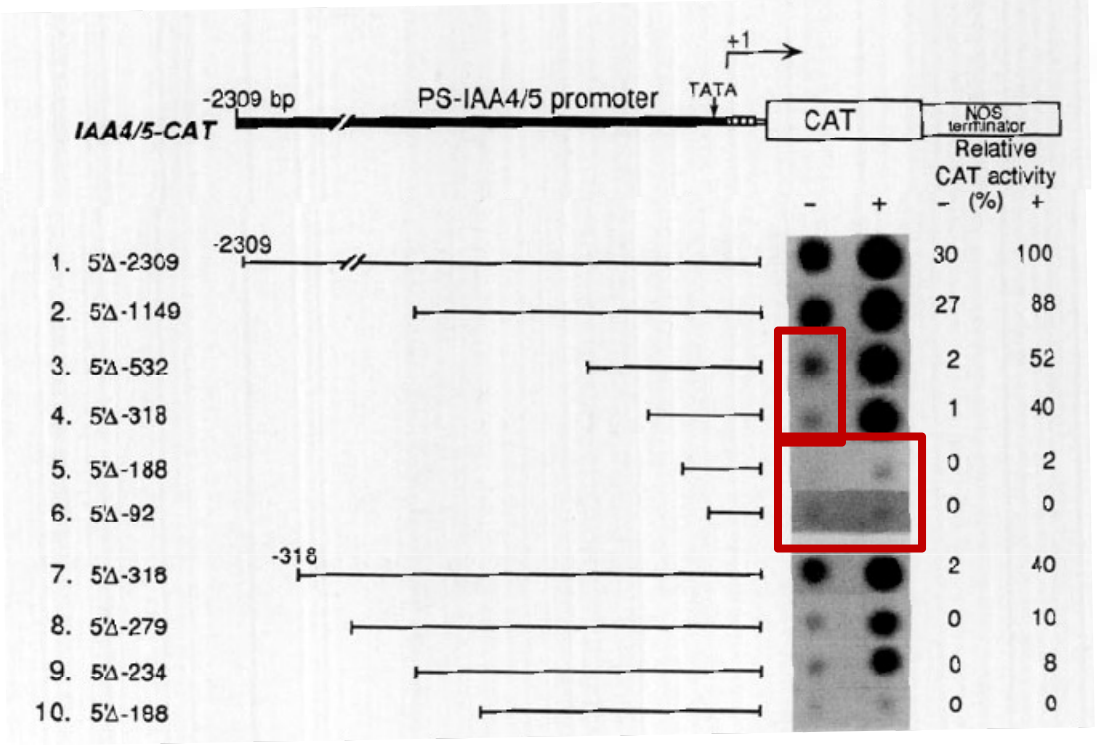




3 Transkriptionsstarts identifiziert:
93, 95 und 99 bp vor dem Start ATG

Experiment wurde auch mit Primer Extension aus dem chimären Konstrukt (*IAA4/5:CAT*) wiederholt = gleiches Ergebnis

promoter deletion analysis



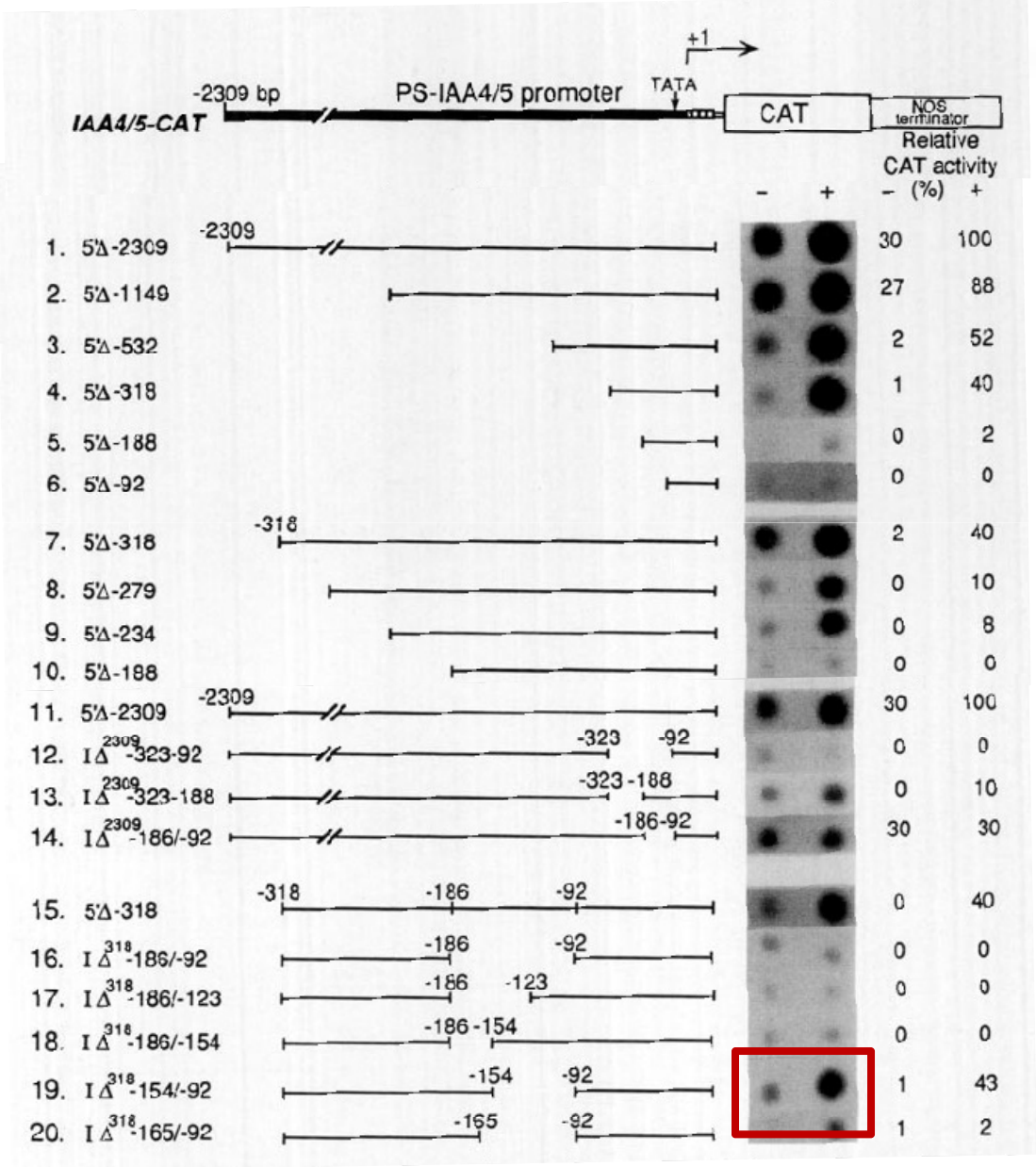
basale CAT Aktivität vermindert - aber Auxin response funktioniert noch

keine/kaum CAT Aktivität

CAT Aktivität

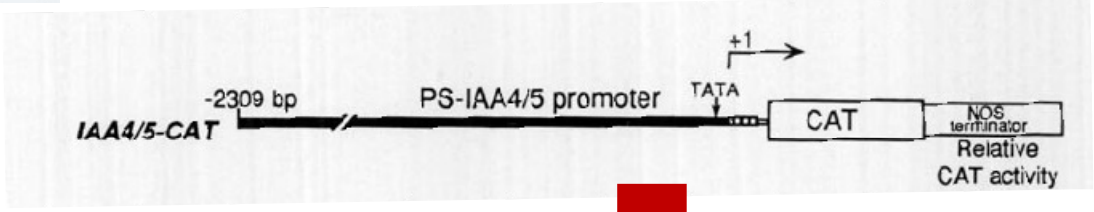
Vom 5´Ende her markiert Position -318 die Grenze zur Position des AuxRE

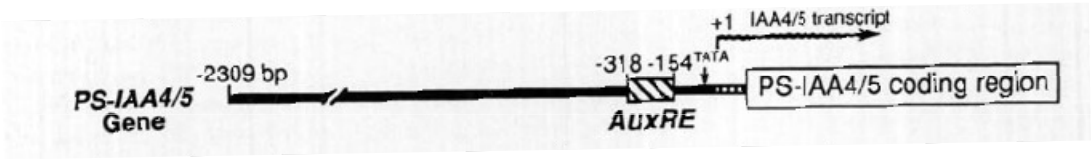
promoter deletion analysis



← -154 markiert 3´Ende des AuxRE

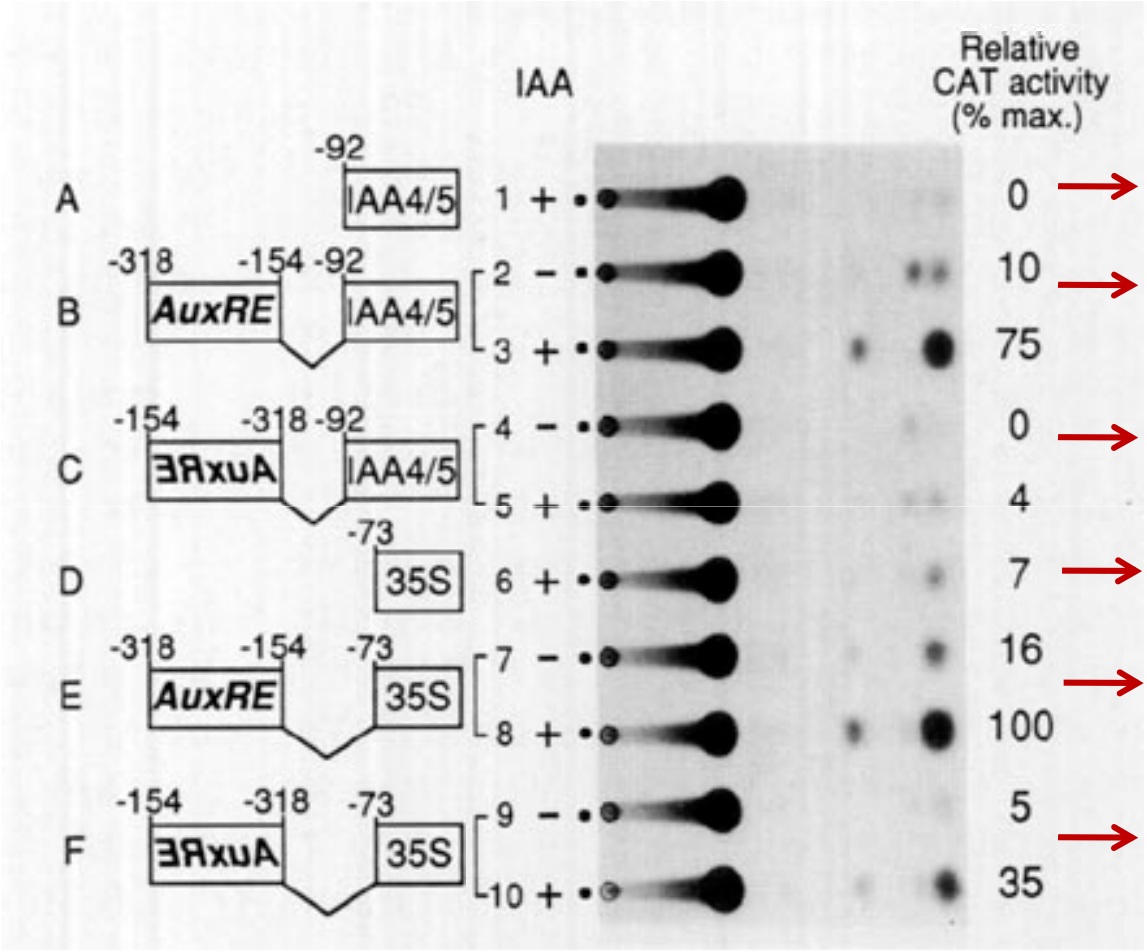
promoter deletion analysis





Das AuxRE liegt im Bereich von -154 bis -318

Testen der “core” sequence



min.Promotor = keine CAT Aktivität

AuxRE = Auxin-induzierter Anstieg der CAT Aktivität

inv.AuxRE = geringe Induktion der CAT Aktivität

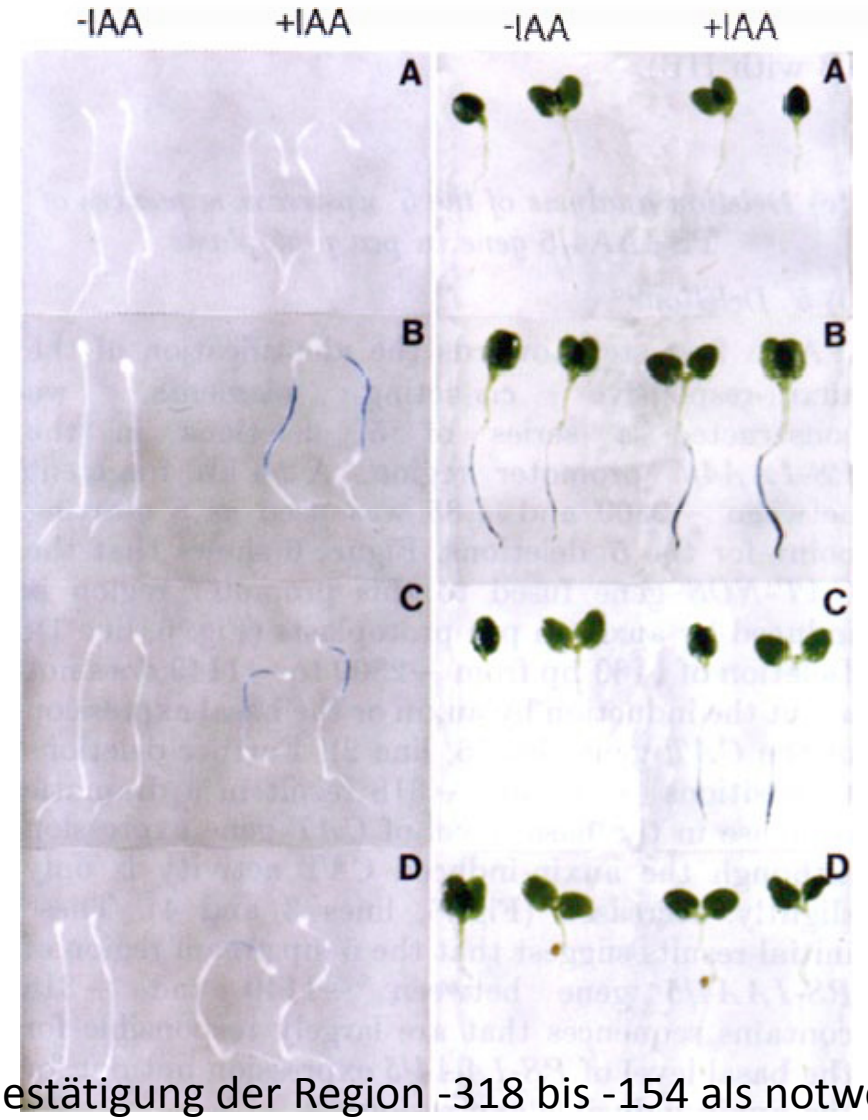
min.Promotor = kaum CAT Aktivität

AuxRE = Auxin-induzierter Anstieg der CAT Aktivität

inv.AuxRE = geringe Induktion der CAT Aktivität

(-318 AuxRE -154) reicht für die Auxin response aus – korrekte Orientierung ist essentiell!

in vivo reporter assay



Konstrukte in Tabak transformiert

GUS

-2309

IAA4/5 Promotor | *GUS*

-318

IAA4/5 Prom. | *GUS*

-188

IAA4/5 | *GUS*

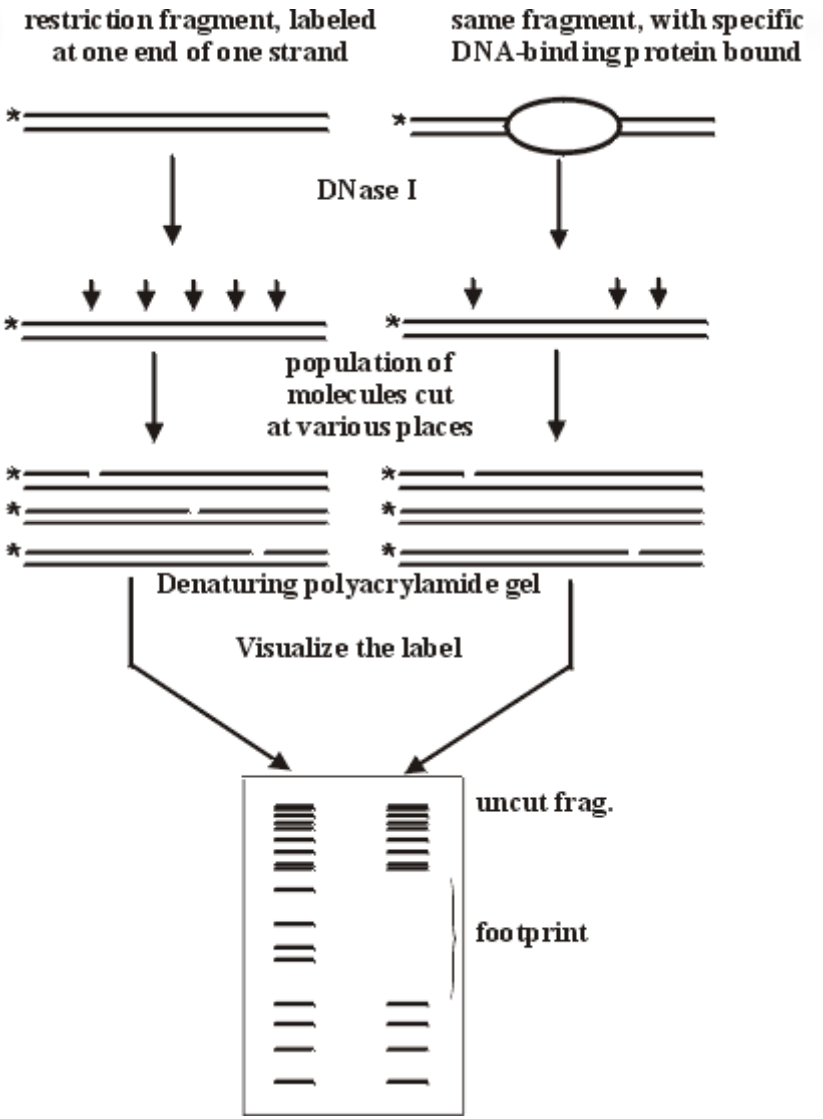
Bestätigung der Region -318 bis -154 als notwendig für die Auxin response
+ Faktoren die dafür in Erbse nötig sind finden sich auch in Tabak

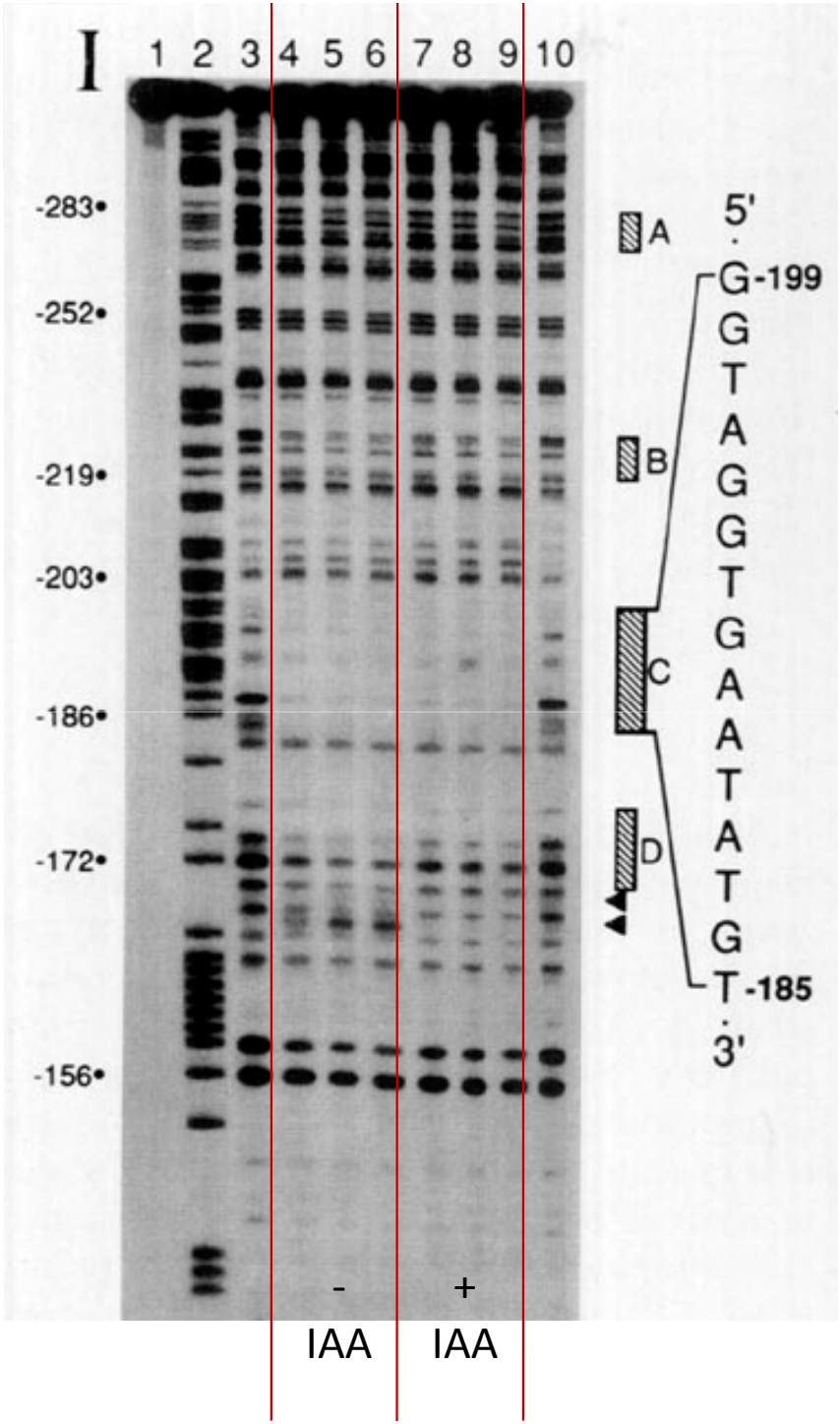
DNase I footprinting

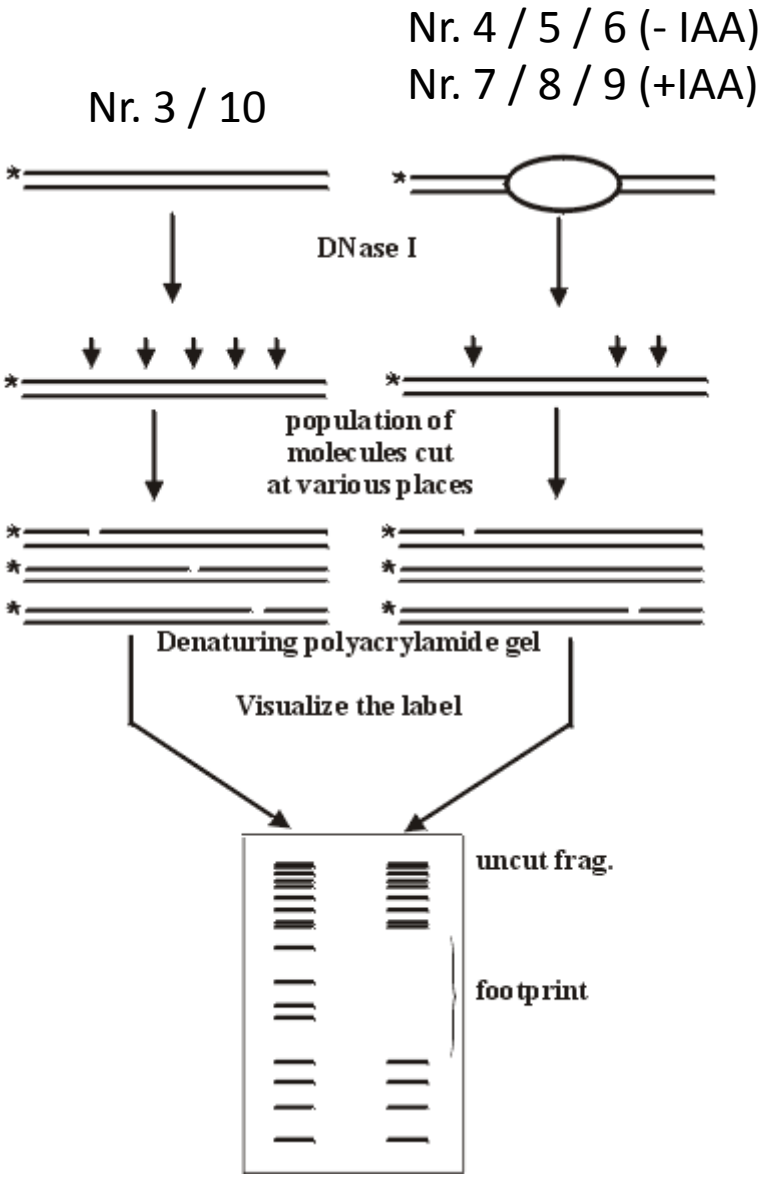
Bereich wurde auf 164 bp eingegrenzt (-318 bis -154)

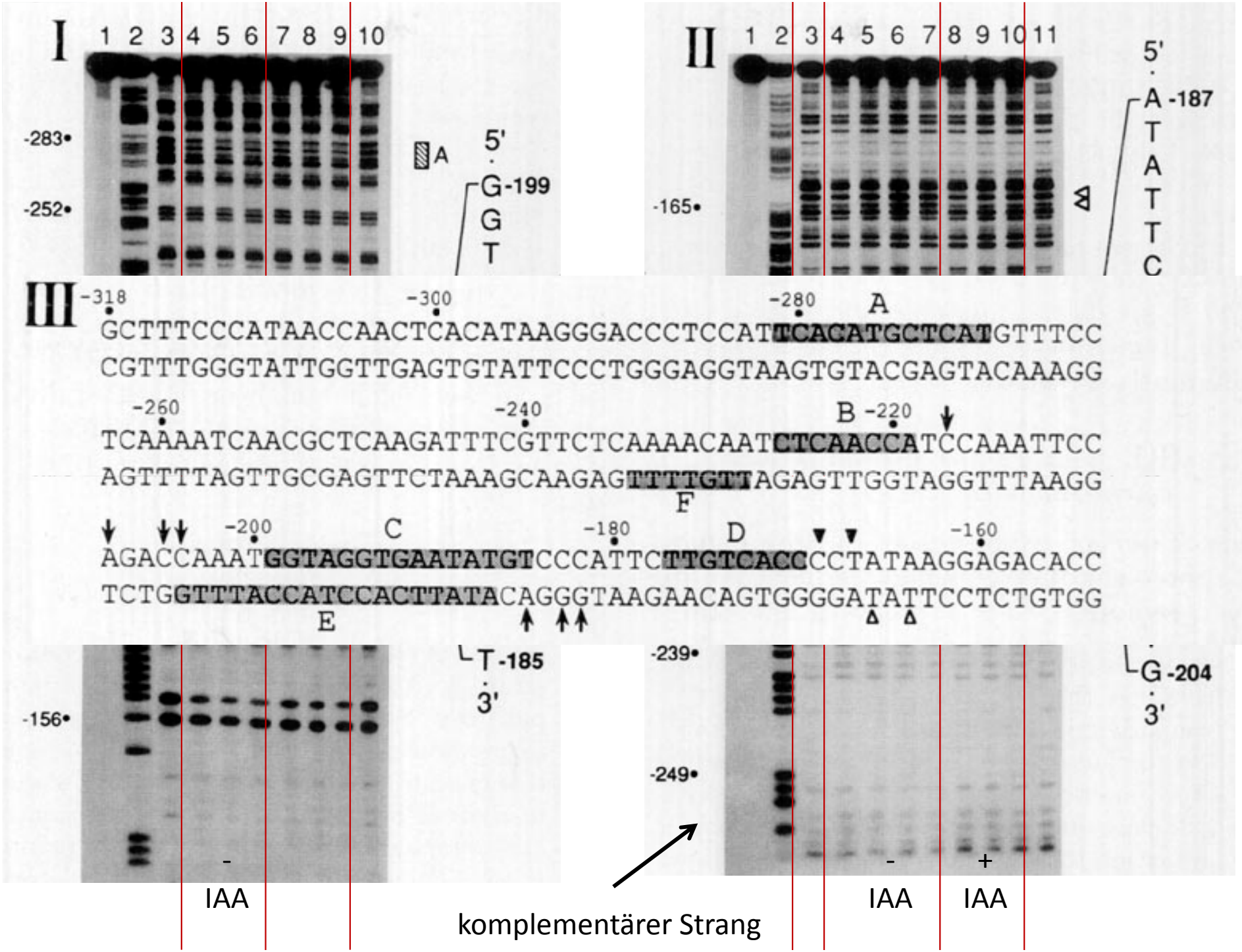
Liegen Bindestellen für kernlokalisierte Proteine (Transkriptionsfaktoren) in diesem Bereich?

DNase I footprinting



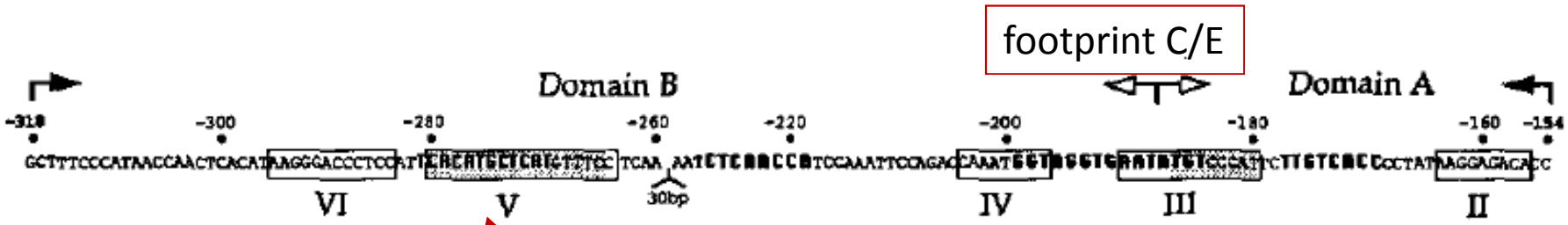

I
1 2 3 4 5 6 7 8 9 10
-283•
-252•
-219•
-203•
-186•
-172•
-156•
A
B
C
D
5'
G-199
GTAGGTGAATATG
T-185
3'
-
+
IAA
IAA


Nr. 4 / 5 / 6 (- IAA)
Nr. 7 / 8 / 9 (+IAA)
Nr. 3 / 10
DNase I
population of
molecules cut
at various places
Denaturing polyacrylamide gel
Visualize the label
uncut frag.
footprint


I
1 2 3 4 5 6 7 8 9 10
-283•
-252•
A
5'
G -199
G
T
II
1 2 3 4 5 6 7 8 9 10 11
-165•
A
5'
A -187
T
A
T
T
C
III
-318
-300
-280
A
GCTTTCCCATAACCAACTCACATAAGGGACCCTCCATTCACATGCTCATGTTTCC
CGTTTGGGTATTGGTTGAGTGTATTCCCTGGGAGGTAAGTGTACGAGTACAAAGG
-260
-240
B -220
TCAAAATCAACGCTCAAGATTTCGTTCTCAAAACAATCTCAACCATCCAAATTCC
AGTTTTAGTTGCGAGTTCTAAAGCAAGAGTTTTGTTAGAGTTGGTAGGTTTAAGG
F
-200
C
-180
D
-160
AGACCAAATGGTAGGTGAATATGTCCCATTCTTGTCACCCTATAAGGAGACACC
TCTGGTTTACCATCCACTTATACAGGGTAAGAACAGTGGGGATATTCCTCTGTGG
E
T -185
3'
-156•
-
IAA
komplementärer Strang
-239•
-249•
-
+
IAA
IAA
G -204
3'

Struktur des AuxRE

II-VI: five sequence motifs identified by Oeller et al. (1993)



CACATGCT**CATGT**TTC

palindrome sequence

TGTCCCAT

(1993)

found in: auxin-responsive genes of *P. sativum*, soybean and *A. thaliana*

Approaches to Dissect Auxin Signaling

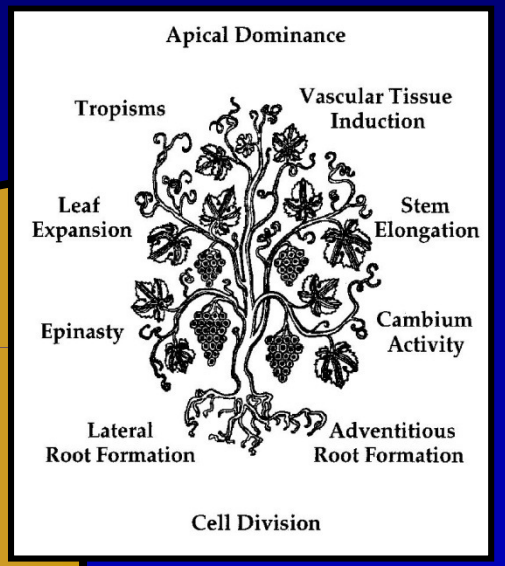
Auxin-related Mutants

Late Genes

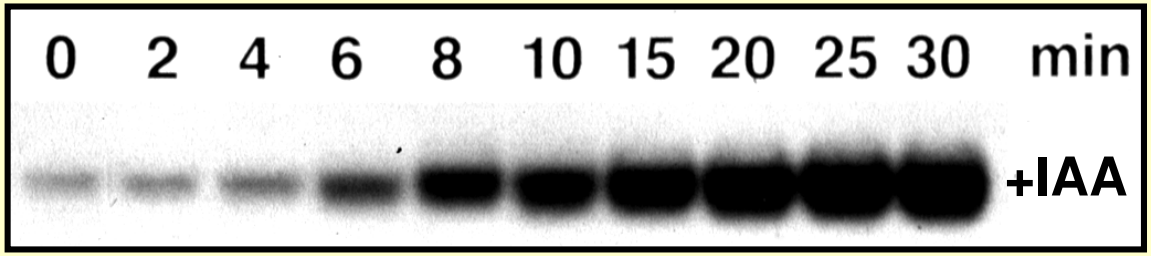
Early Genes

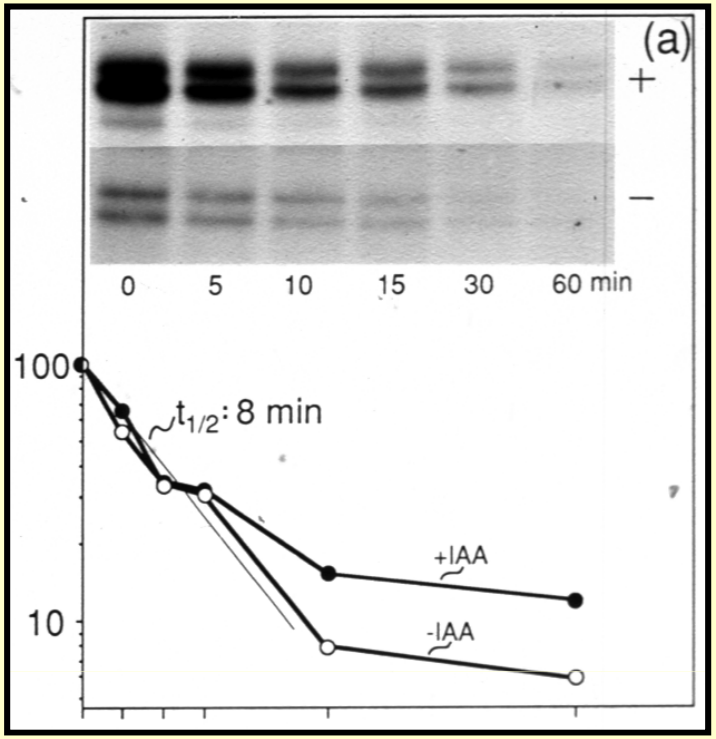
Receptor

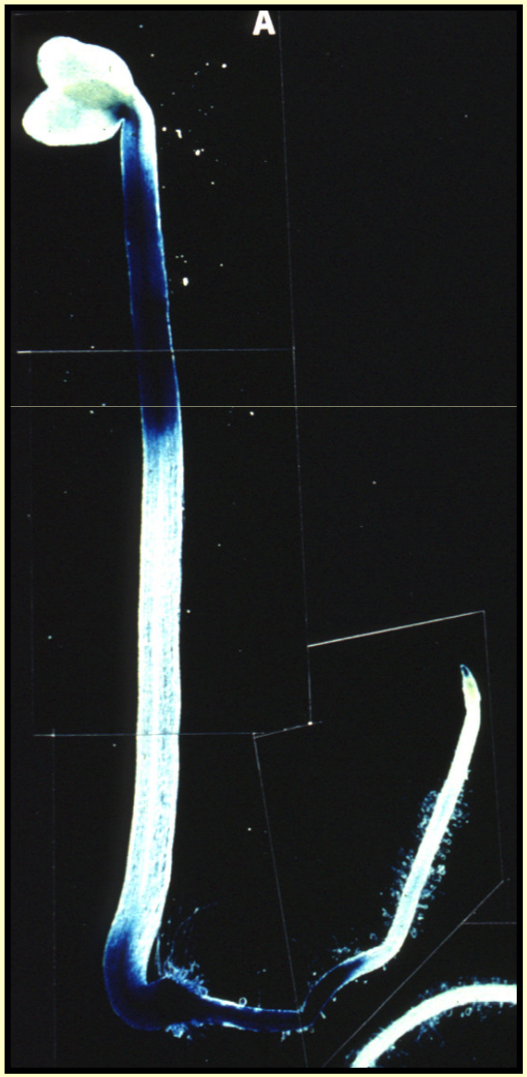
Auxin



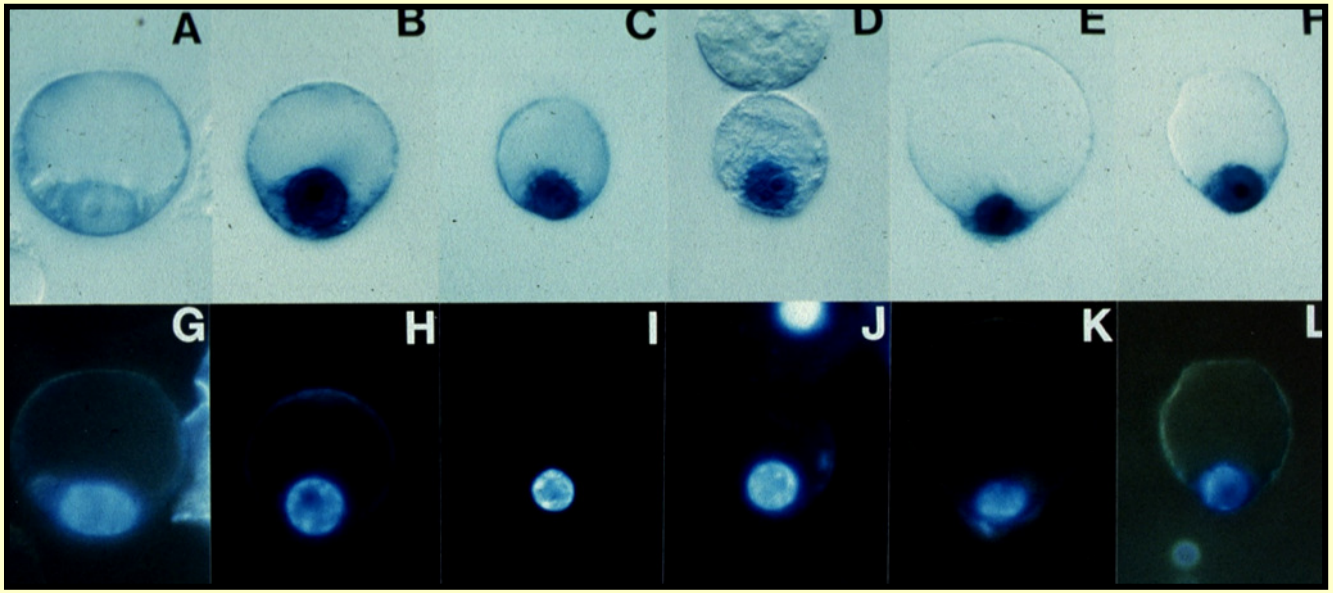
AuxRE ↔ Proteins





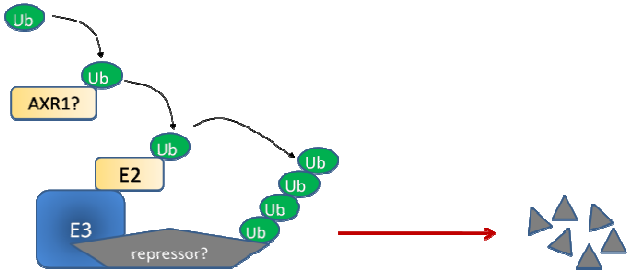


Highlights of *Aux/IAA* Gene Expression



Modelle (1993/94)

Short-lived nuclear proteins (possibly transcription factors)



auxin response