

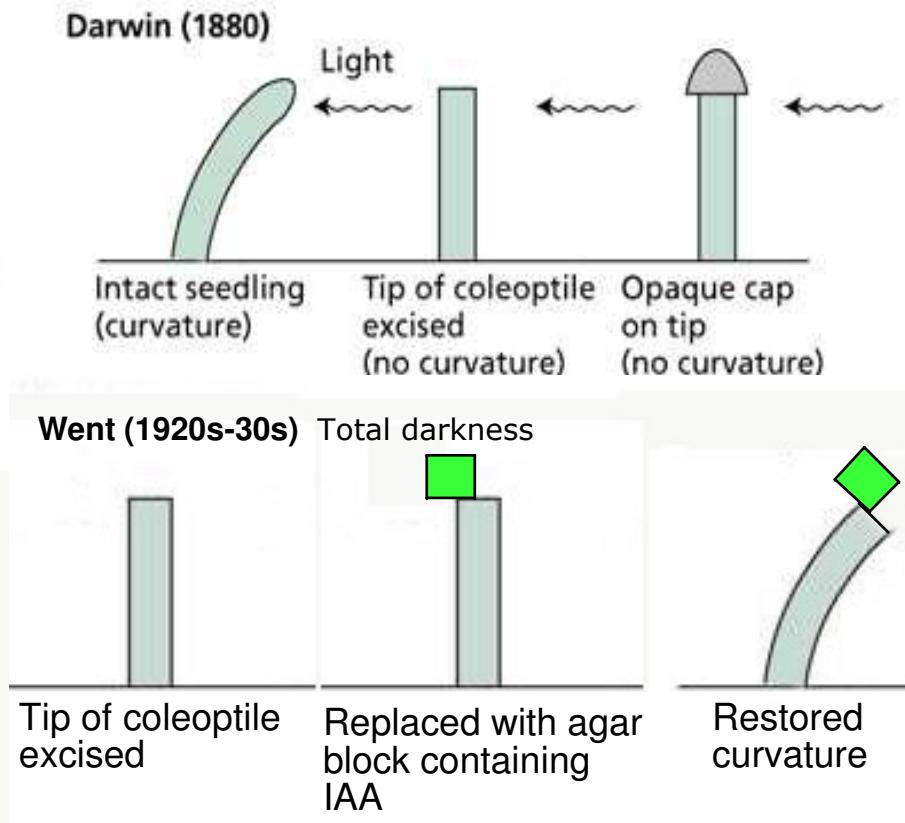
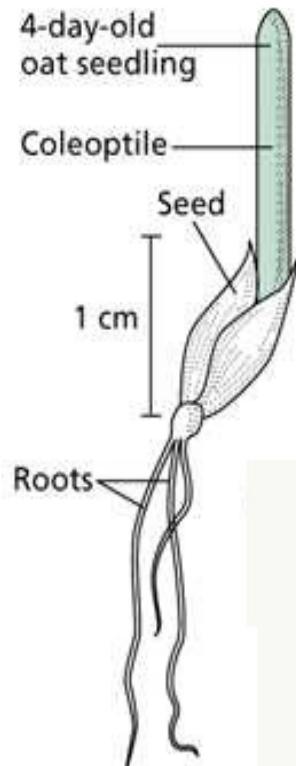
Seminar

Molekulare Mechanismen der Signaltransduktion

Gliederung (22.04.2009):

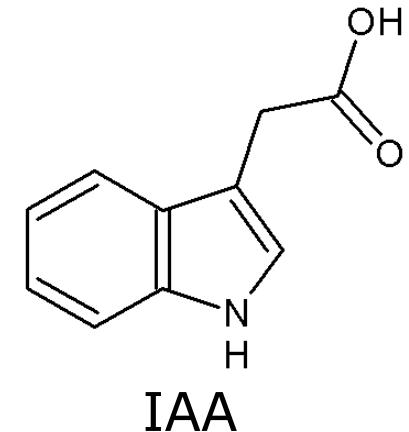
- ✓ Allgemeine Einleitung (Auxin, Nomenklatur....)
 - ✓ Vorstellung der ersten paper
 - ✓ Termine verteilen
-
1. Estelle and Somerville, (1987) Auxin resistant mutants of *Arabidopsis thaliana* with an altered morphology. **MGG** 206:200
 2. Lincoln et al., (1990) Growth and development of the *axr1* mutants of *Arabidopsis*. **PC** 2:1071
 3. Leyser et al., (1993) *Arabidopsis* auxin-resistance gene *AXR1* encodes a protein related to ubiquitin-activating enzyme E1. **N** 364:161

Auxin - history and pioneering experiments



"When seedlings are freely exposed to a lateral light some influence is transmitted from the upper part of the coleoptile that acts on the lower part of the coleoptile"

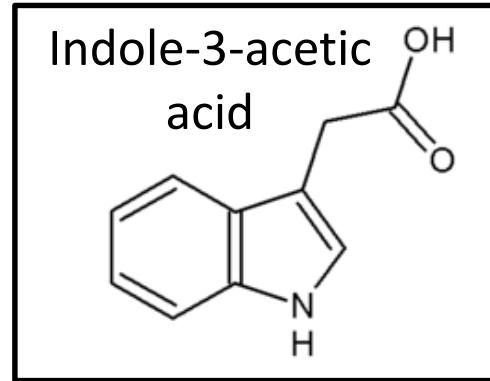
"The Power of Movement in Plants" (1880) by Darwin and Darwin.



- *auxein* (greek) = to grow
- first phytohormone to be identified

indole-3-acetic acid

Auxin regulates plant development

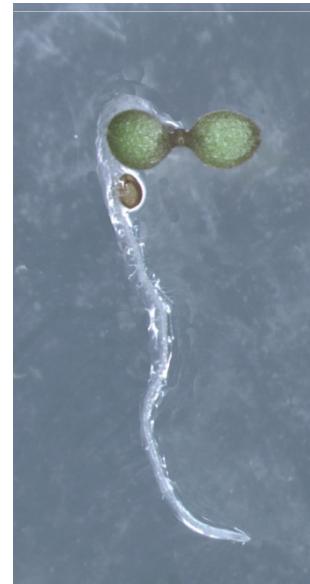


→ Embryonic patterning

Growth & Apical dominance

Root development

Tropic growth responses

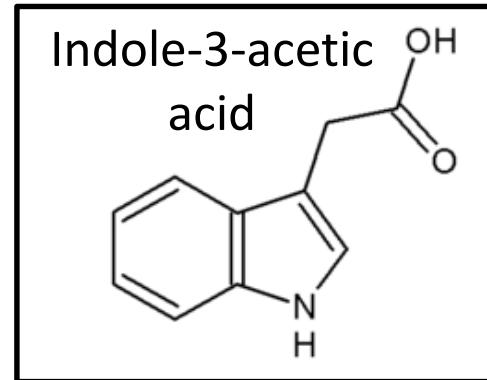


wild-type



bdl axr1 mutant

Auxin regulates plant development



Embryonic patterning

→ Growth & Apical dominance

Root development

Tropic growth responses

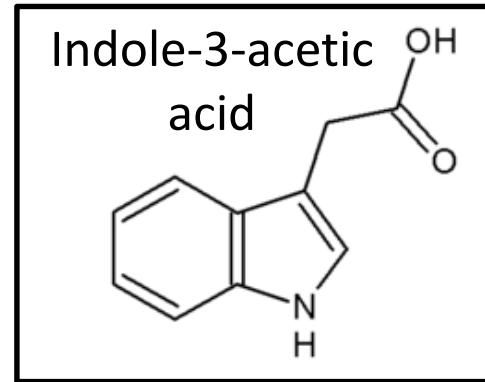


wild-type



axr6-3 mutant

Auxin regulates plant development

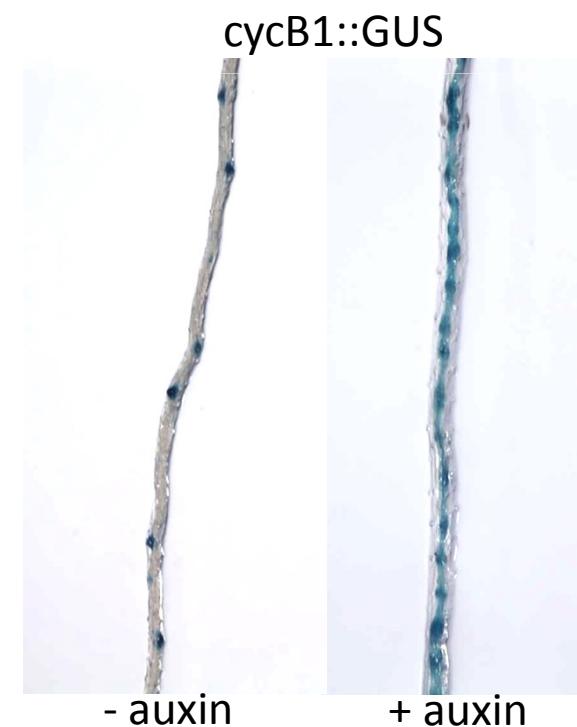


Embryonic patterning

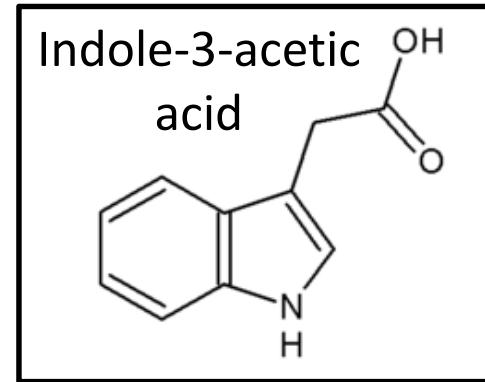
Growth & Apical dominance

→ Root development

Tropic growth responses



Auxin regulates plant development

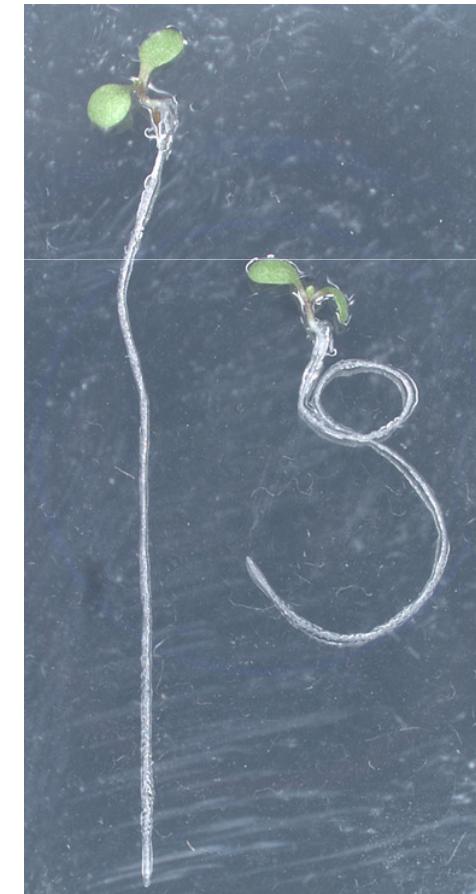


Embryonic patterning

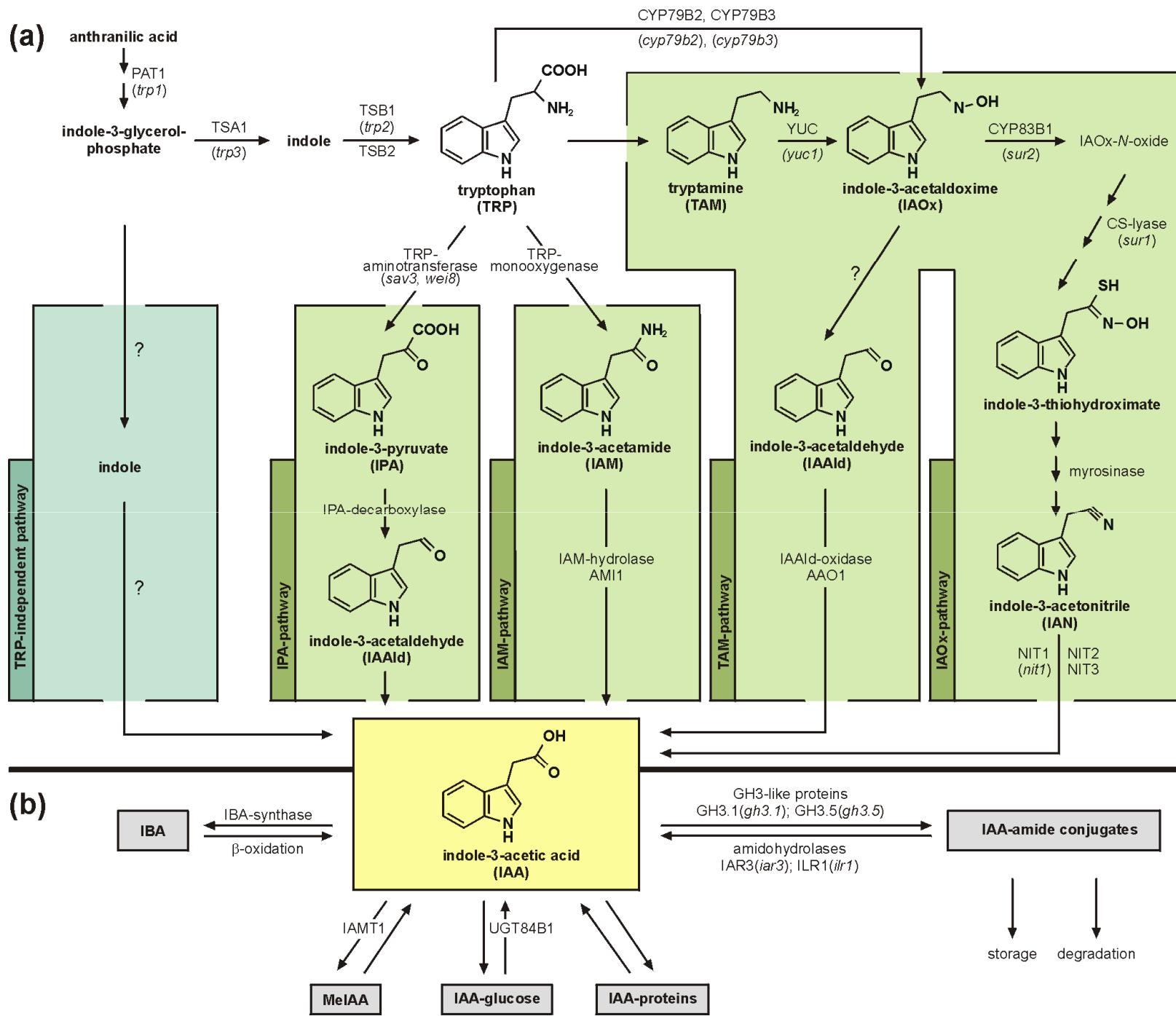
Growth & Apical dominance

Root development

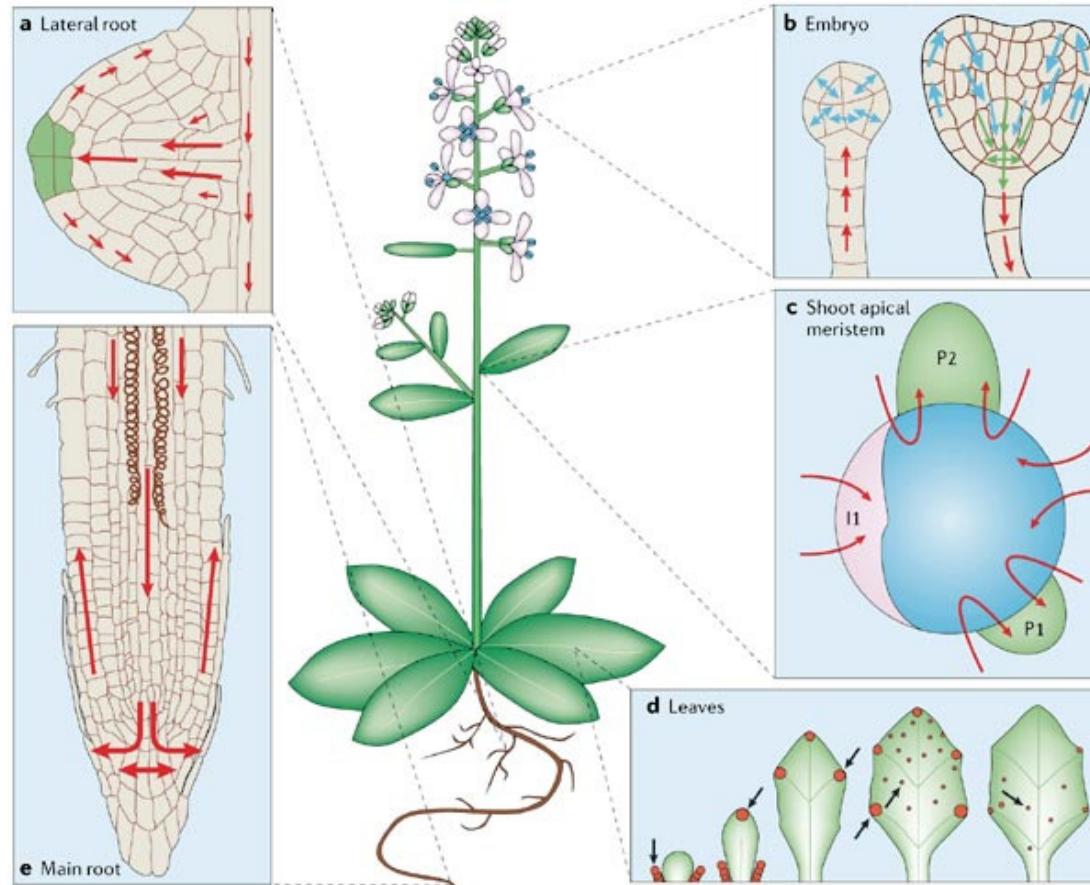
→ Tropic growth responses



wild-type *aux1* mutant

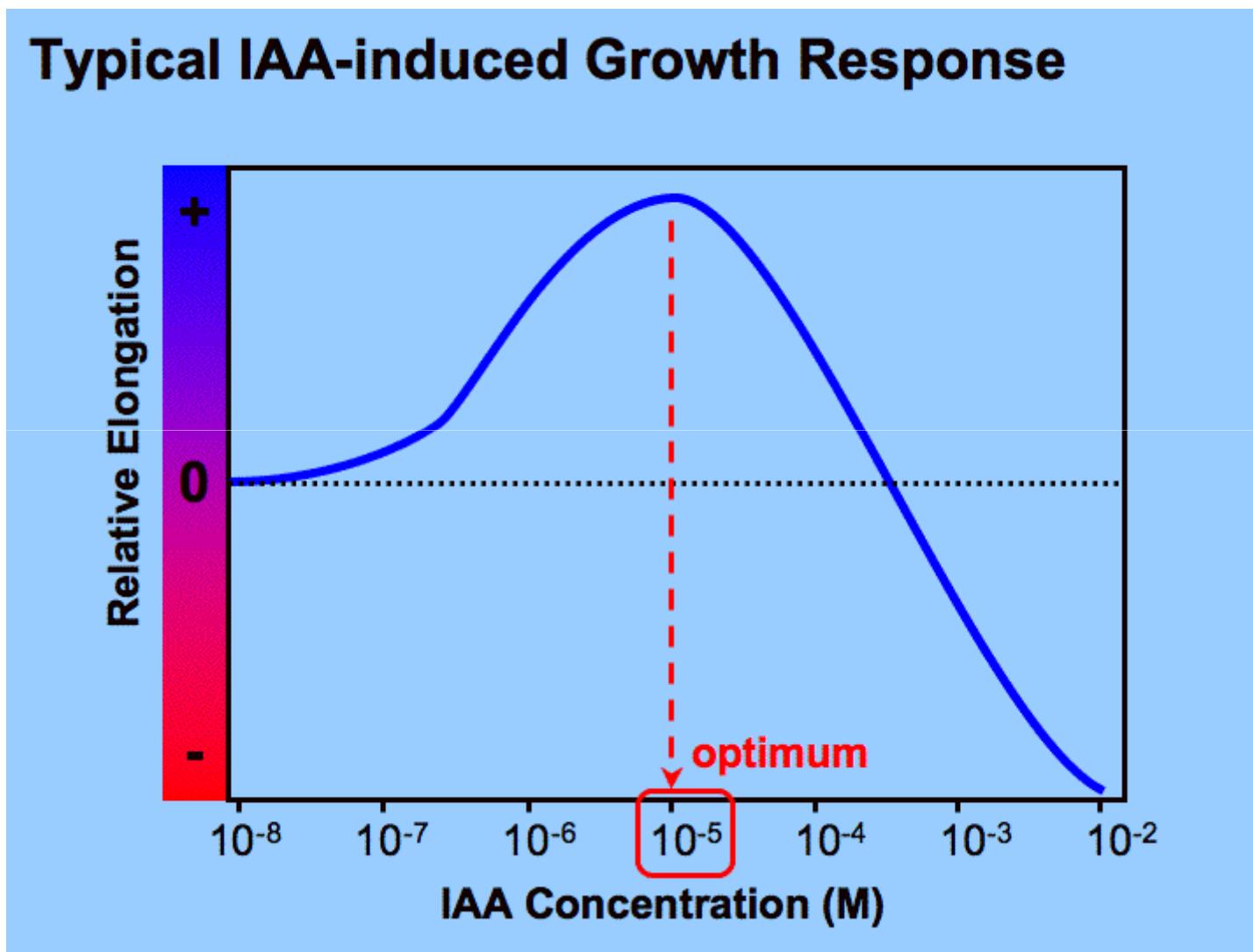


Auxintransport



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Auxin response (Einfluss auf das Wurzelwachstum)



Arabidopsis thaliana



- Small size (30 cm)
- Rapid life cycle (6 weeks)
- Prolific seed production (5000 seeds/plant)
- Sequenced genome (125 Mb; ~26,000 genes)
- Easily transformable
- Tremendous community resources

A power multicellular eukaryotic model system

Allgemeines zur Nomenklatur

3 Buchstaben Code:

leitet sich meist von einer Mutante / Phänotyp ab (z.B:
altered axin response 1)

- **AXR1** - Gen oder Transkript (=mRNA)
- **AXR1** - Protein
- **axr1** - Mutante mit Defekt im AXR1 Gen
- **axr1-1, axr1-2, axr1-3....** - verschiedene Mutanten des AXR1 Gens (= verschiedene Allele)
- **axr1, axr2, axr3....** - Mutanten mit Defekten in verschiedenen Genen

Allgemeines zum paper Aufbau / zur paper Präsentation

- Abstract
 - Zusammenfassung des papers
- Introduction
 - Einleitung / Vorstellung der Hintergründe
- Materials/Methods
 - was wurde wie gemacht
- Results
- Diskussion
 - Ergebnisse werden evaluiert und in Zusammenhang mit Daten aus der Literatur gebracht
- Literature cited
- Hintergrund / Einleitung /**Ausgangsmodell**
- Zielstellung!
- Ergebnisse (anhand der Abbildungen)
- Diskussion
- Fazit: welche Erkenntnisse sind dazu gekommen

Model erweitern

Präsentationen werden nach dem jeweiligen Seminar alle auf die Webseite gestellt!
<http://quintlab.openwetware.org/Teaching.html>

Auxin-resistant mutants of *Arabidopsis thaliana* with an altered morphology

Mark A. Estelle* and Chris Somerville

MSU-DOE Plant Research Laboratory, Michigan State University, East Lansing, MI 48824, USA

Ziel:

Isolierung von Mutanten, die eine erhöhte Resistenz gegenüber Auxin aufweisen

Identifizierung von Signalelementen, die an der Auxinresponse beteiligt sind

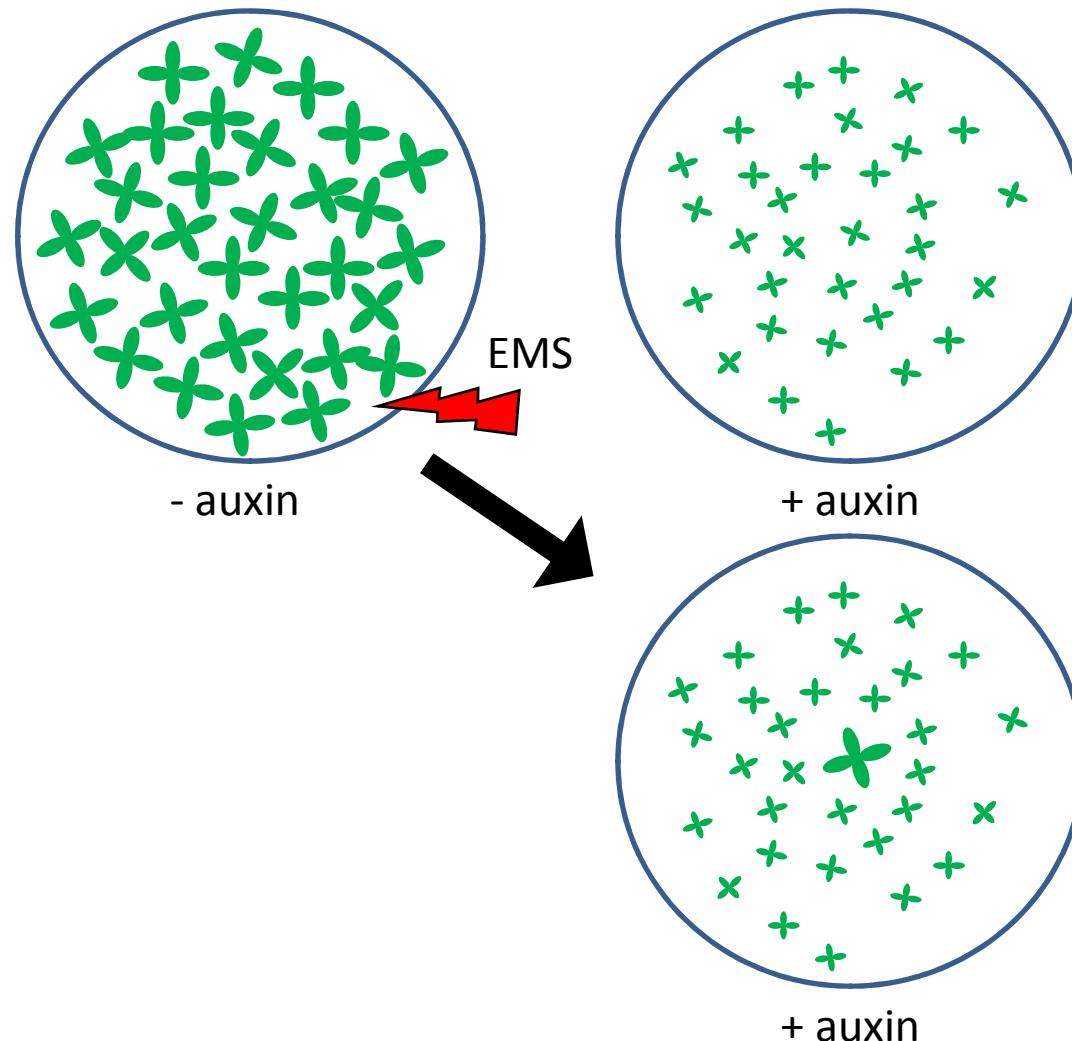
Zu diesem Zeitpunkt:

- keine Enzyme der Auxinbiosynthese bekannt
- keine Information zu Elementen der Signaltransduktion (weder allgemeine noch gewebsspezifische)
- *Arabidopsis* war noch nicht sequenziert!

Mutantenscreen:

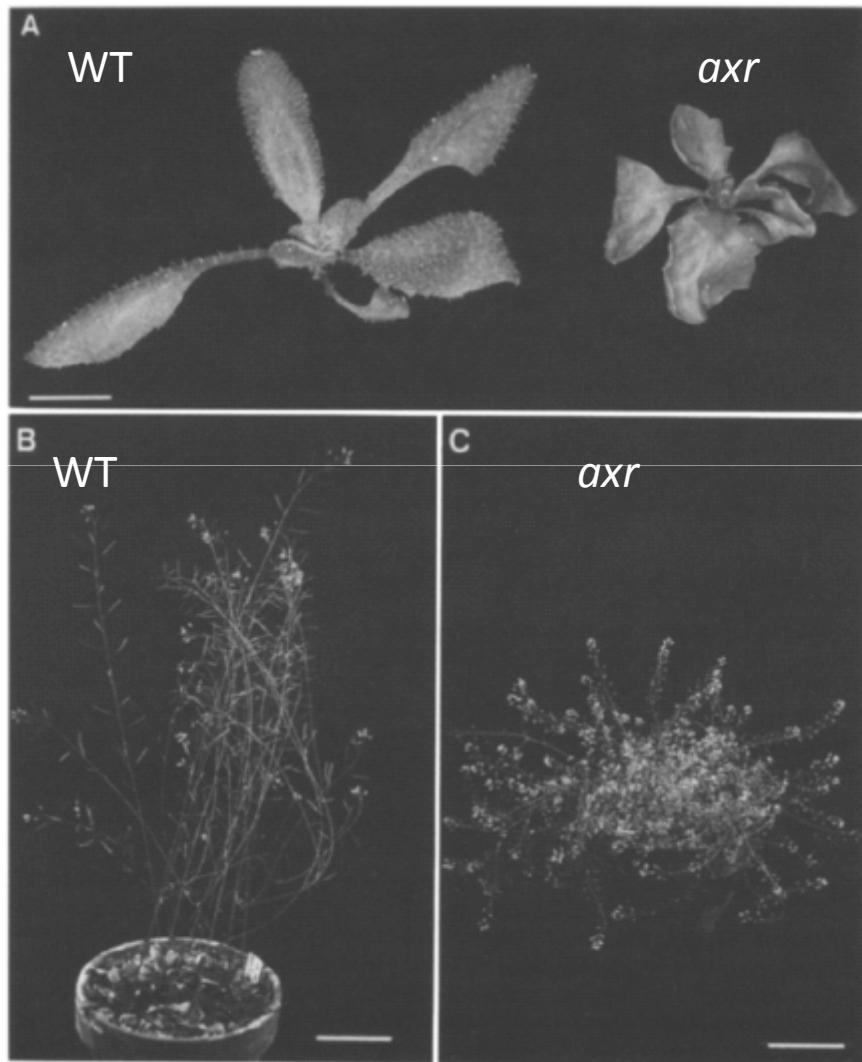
EMS-Mutagenese von Samen

Screen nach Mutanten, die resistent gegenüber der Auxinbehandlung sind



12 *axr* Mutanten
aus 300 000
gescreenten

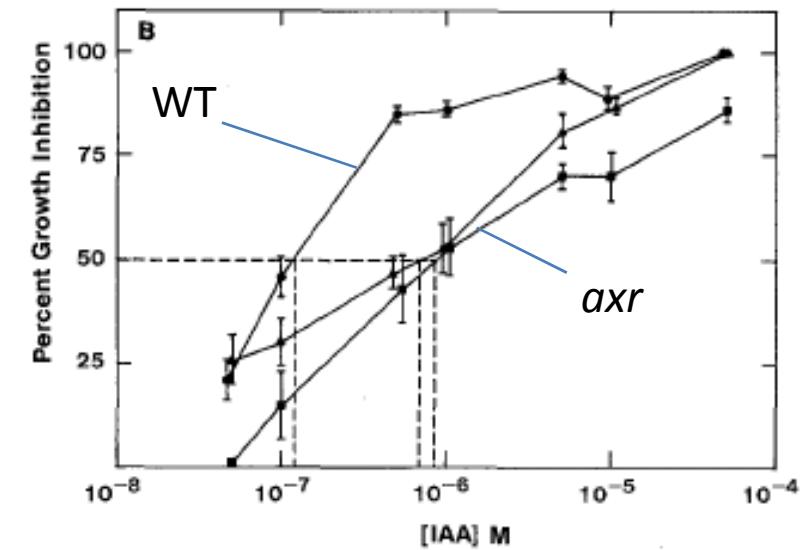
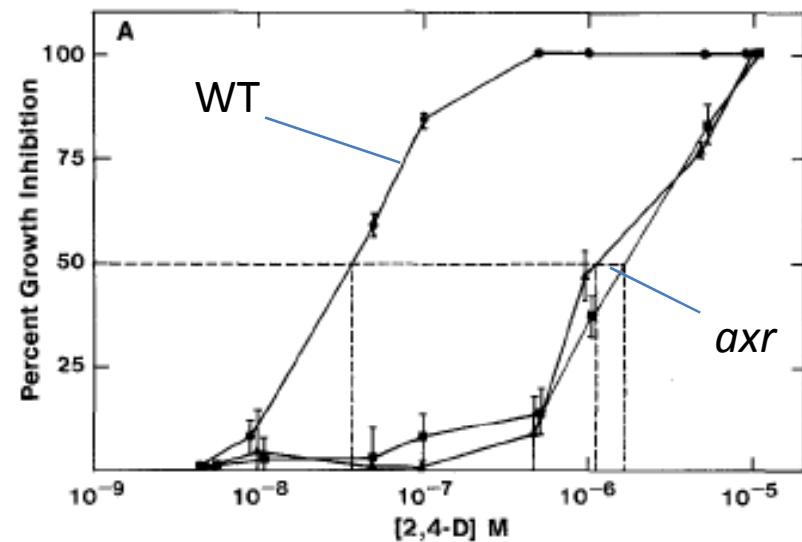
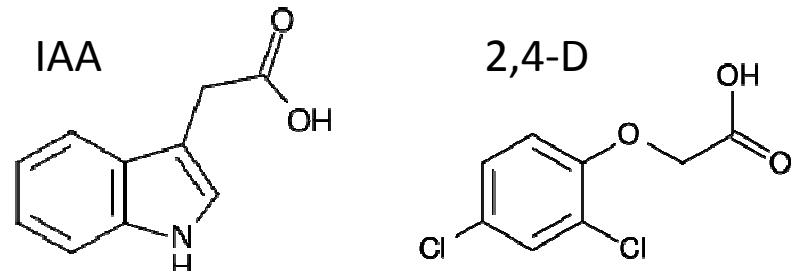
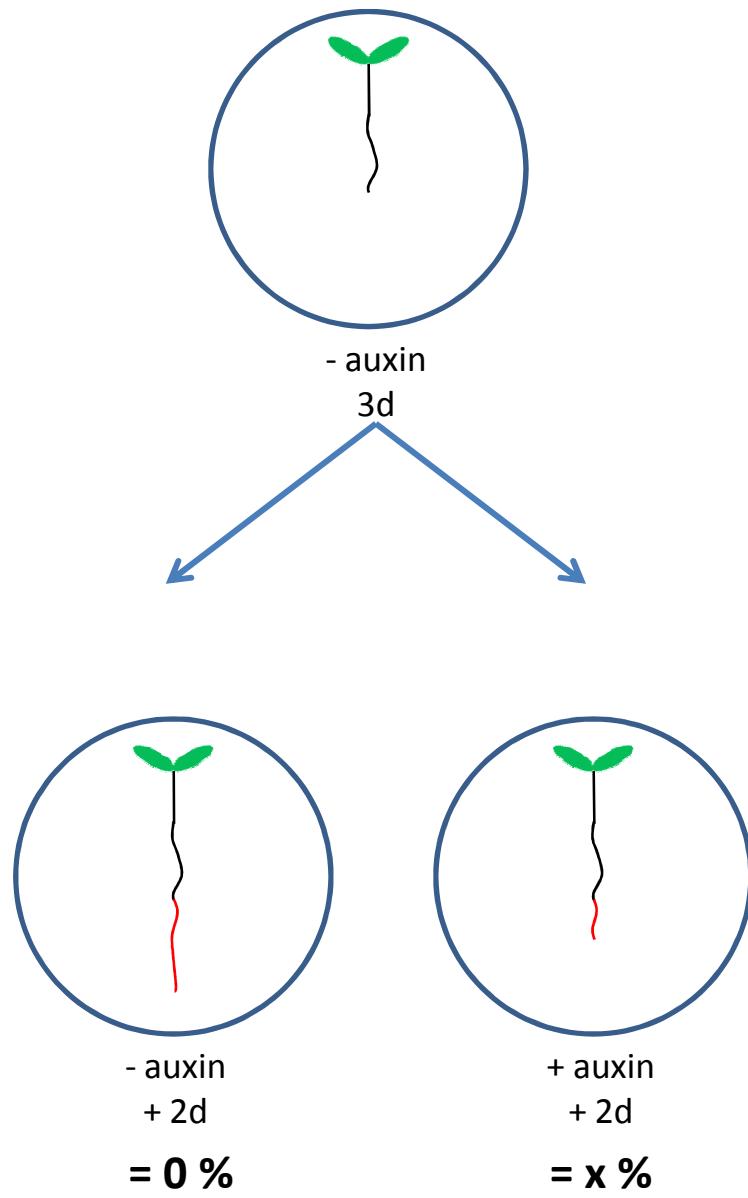
axr = altered axin response



→ *axr1* Mutanten sind:
kleiner (Rosette)
kürzere Blattstiele
Veränderte Blattform

→ *axr1* Mutanten bilden vermehrt
sekundäre Infloreszenzen
= Verlust der Apikaldominanz

2. Wurzelelongation auf Auxin



Auxin-resistant mutants of *Arabidopsis thaliana* with an altered morphology

Mark A. Estelle* and Chris Somerville

MSU-DOE Plant Research Laboratory, Michigan State University, East Lansing, MI 48824, USA

Fazit:

Isolierung von *axr1* Mutanten mit erhöhter Resistenz gegenüber Auxin, die außerdem zahlreiche Wachstums- und Entwicklungsphänotypen zeigen

eine mögliche Hypothese:

“....An attractive possibility is that the *AXR1* gene coded for an auxin receptor and that resistance is due to an alteration that has a greater effect on the affinity of this receptor for 2,4-D than for IAA.....”

Growth and Development of the *axr1* Mutants of *Arabidopsis*

Cynthia Lincoln, James H. Britton, and Mark Estelle¹

Department of Biology, Indiana University, Bloomington, Indiana 47405

Ziel:

Weitere Charakterisierung der *axr1*-Mutanten
Kartierung des betroffenen Gens

Table 1. Recovery of *axr1* Mutants^a

M2 population	Mutagen	Selection	Mutants recovered
A ^b	EMS	2,4-D	<i>axr1</i> -1
			<i>axr1</i> -2
			<i>axr1</i> -3
			<i>axr1</i> -4
			<i>axr1</i> -5
			<i>axr1</i> -6
B ^b	EMS	2,4-D	<i>axr1</i> -7
			<i>axr1</i> -8
			<i>axr1</i> -9
			<i>axr1</i> -11
			<i>axr1</i> -12
			<i>axr1</i> -15
C ^c	EMS	2,4-D	<i>axr1</i> -16
			<i>axr1</i> -17
			<i>axr1</i> -18
			<i>axr1</i> -19
			<i>axr1</i> -20
			<i>axr1</i> -21
C ^c	EMS	IAA	<i>axr1</i> -22
D ^c	γ	2,4-D	<i>axr1</i> -23

^a A total of 470,000 seeds from four distinct M2 populations was screened for mutants that were able to elongate roots on either 5 μ M 2,4-D or 50 μ M IAA.

^b Estelle and Somerville (1987).

^c This study.

zu den 12 *axr1*-Mutanten
noch weitere 8 isoliert

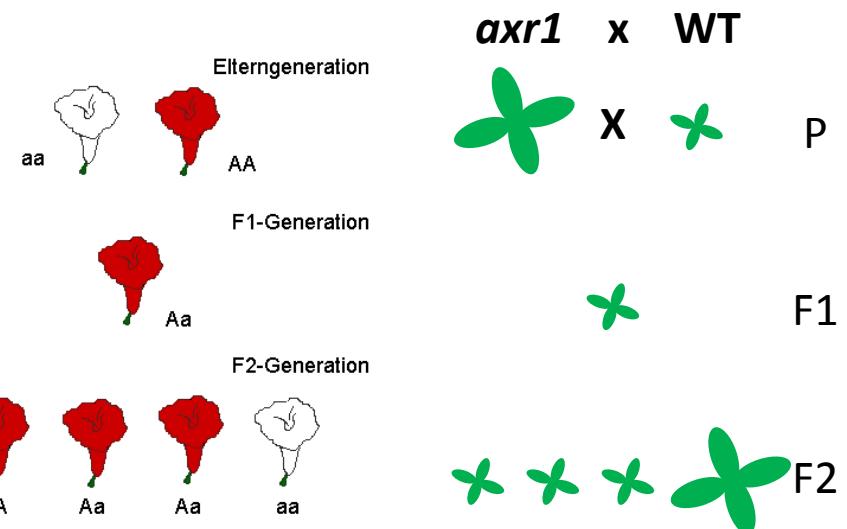


Table 2. Genetic Segregation of 2,4-D Resistance in *axr1* Lines

Cross	Number of Plants			χ^2 *
	Resistant	Sensitive		
<i>axr1</i> -19 x wild-type F1	0	23		
	186	493		2.07 ^b
<i>axr1</i> -21 x wild-type F1	0	51		
	82	281		1.12 ^b
<i>axr1</i> -22 x wild-type F1	0	22		
	56	216		2.83 ^b
<i>axr1</i> -23 x wild-type F1	0	33		
	117	383		0.683 ^b

* χ^2 was calculated based on an expected ratio of three sensitive to one resistant.

^b P > 0.05.



axr1 mutants are recessive

Mutationen allelisch oder in verschiedenen Genen?

Mutationen allelisch oder in verschiedenen Genen?
→ Komplementationstest auf Auxin

axr1-12/axr1-12



axr1-3/axr1-3

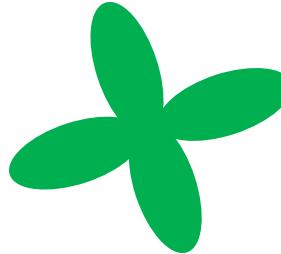
X



F1



oder



axr/AXR

axr/axr

Table 3. Complementation Analysis of *axr1* Lines

Cross	Number of Plants	
	Resistant	Sensitive
<i>axr1-12</i> × <i>axr1-3</i>	33	0
<i>axr1-19</i> × <i>axr1-3</i>	21	0
<i>axr1-20</i> × <i>axr1-3</i>	13	0
<i>axr1-22</i> × <i>axr1-3</i>	24	0
<i>axr1-23</i> × <i>axr1-3</i>	39	0



→ Mutationen sind verschiedene Allele im selben Gen!

Morphologie:

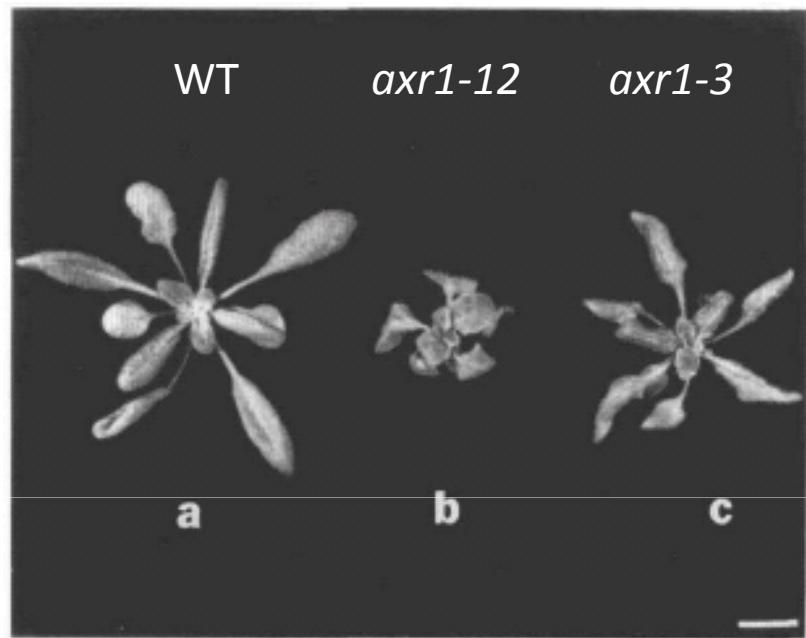


Figure 1. Phenotype of Wild-Type and Mutant Rosettes.

Rosettes were photographed when the plants were 3 weeks old.

- (a) Wild type.
- (b) *axr1-12/axr1-12*.
- (c) *axr1-3/axr1-3*.

Bar = 1 cm.

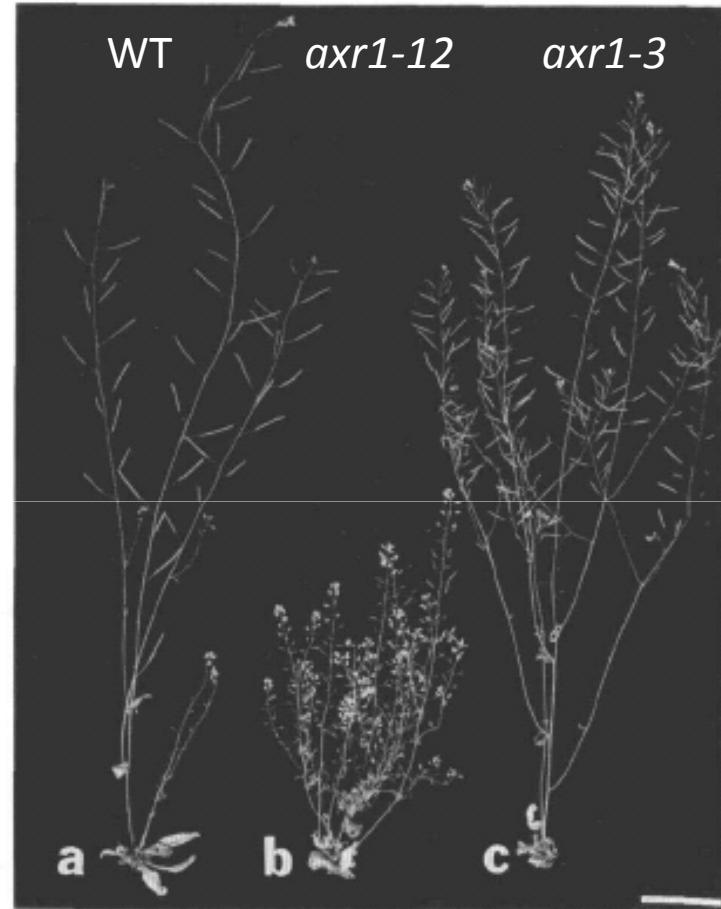


Figure 2. Comparison of Mature Wild-Type and Mutant Plants.

Wild-type and mutant plants were photographed when 7 weeks old.

- (a) Wild type.
- (b) *axr1-12/axr1-12*.
- (c) *axr1-3/axr1-3*.

Bar = 3 cm.

→ Allele zeigen unterschiedliche Ausprägung morphologischer Defekte

Quantifizierung morphologischer Unterschiede:

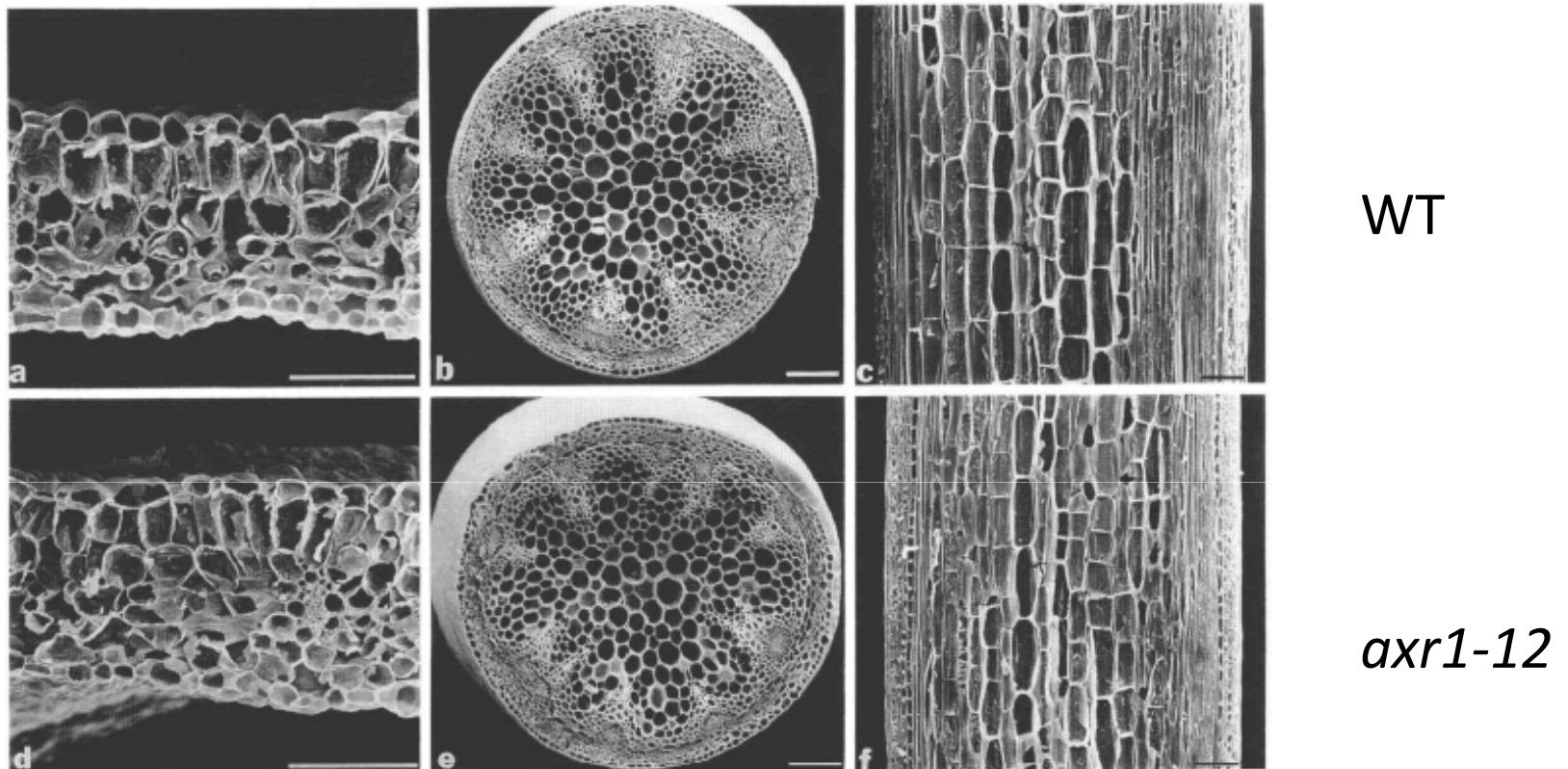
Table 4. Morphology of Wild-Type and Mutant Plants

	Wild Type	<i>axr1-3</i>	<i>axr1-12</i>
Height (cm)	50.9 ± 1.0	38.4 ± 1.3	19.2 ± 1.8
No. of inflorescences	5.75 ± 0.25	6.25 ± 1.4	6.6 ± 1.6
No. of lateral branches	46.2 ± 4.5	96.7 ± 21.4	124.0 ± 24.4
Distance between siliques (cm)	0.65 ± 0.04	0.30 ± 0.01	0.32 ± 0.02
No. of siliques	673.75 ± 68.0	526.5 ± 56.0	2.4 ± 1.3
No. of pollen grains/flower	2035.0 ± 500.0	2700.0 ± 337.0	680.0 ± 32.0
Hypocotyl length in etiolated seedlings (cm)	1.40 ± 0.04	1.20 ± 0.05	0.77 ± 0.03



axr1-3 Mutante zeigt eine schwächere Ausprägung der Phänotypen als die *axr1-12* Mutante

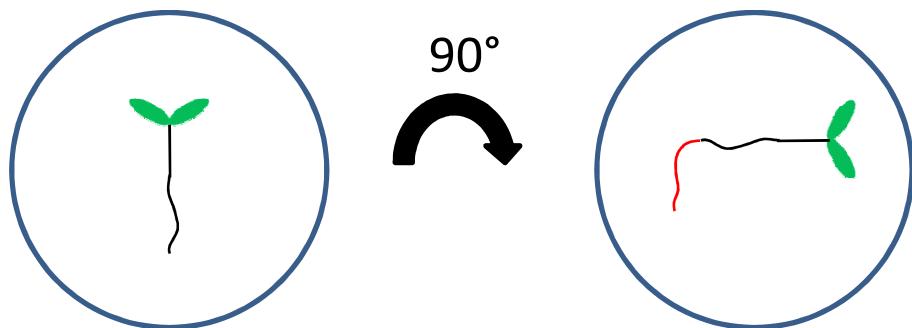
SEM → morphologische Defekte durch unterschiedliche Zellgrößen oder Gewebeorganisation?



- keine wesentlichen strukturellen Defekte
- vaskuläre Strukturen etwas weniger differenziert
- Zellgrößen in etwa gleich = Wachstumsreduktion durch
reduzierte Zellteilung

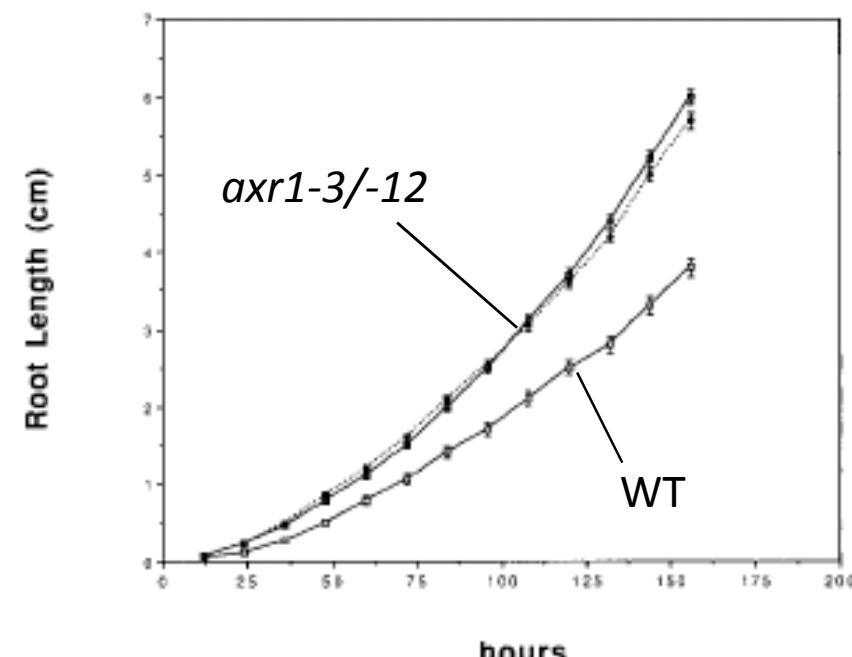
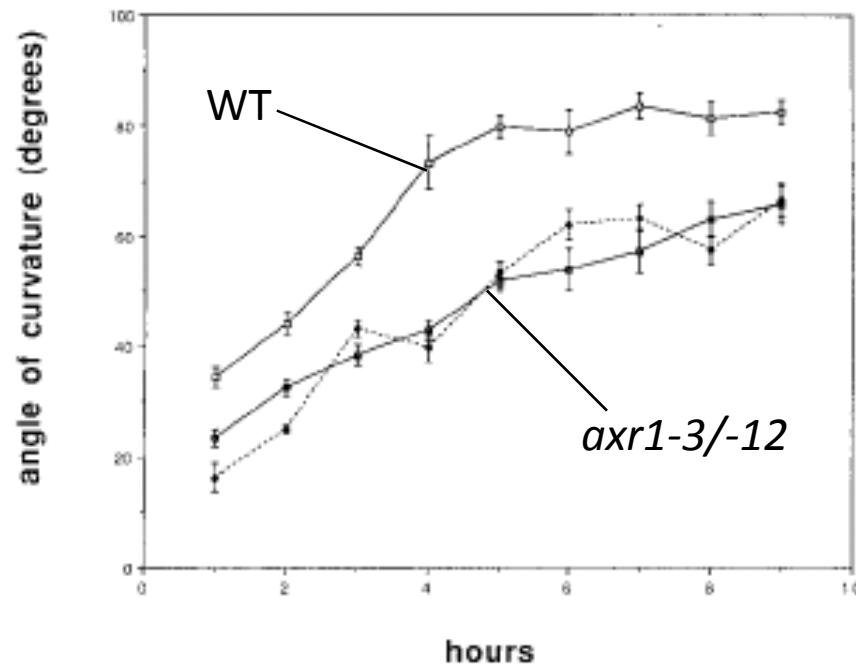
Auxin Response in der Wurzel:

1. Gravitropismus



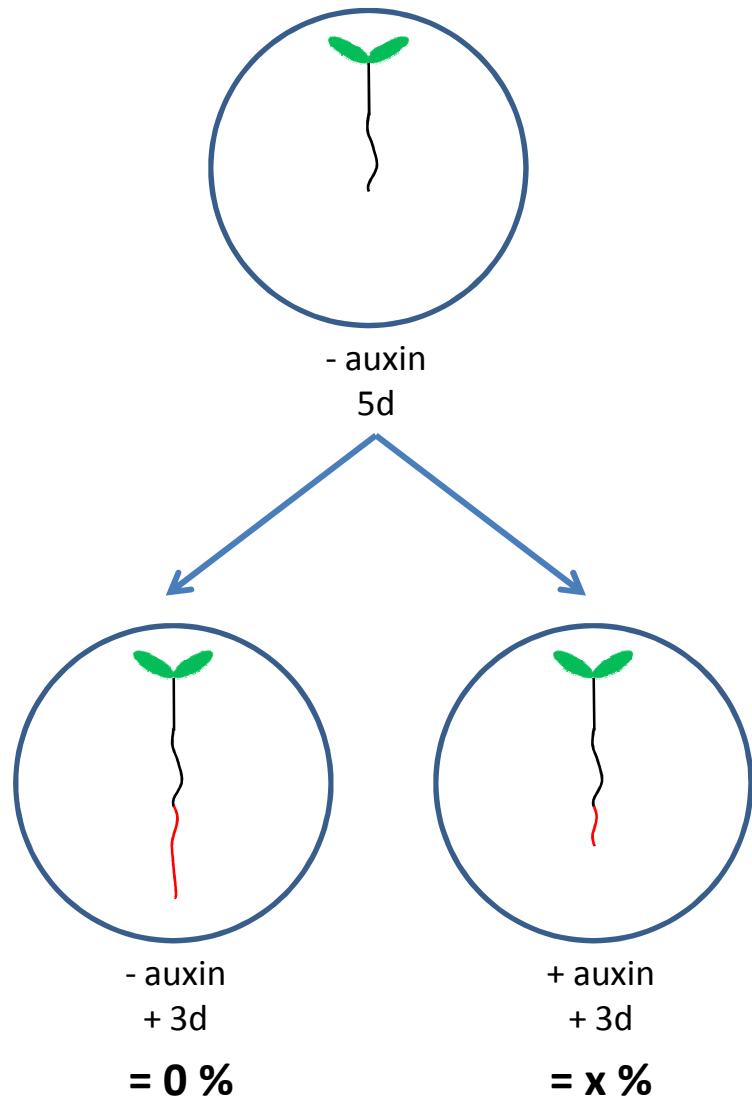
→ Graviresponse in den Mutanten langsamer

→ nicht durch reduziertes Wurzelwachstum!

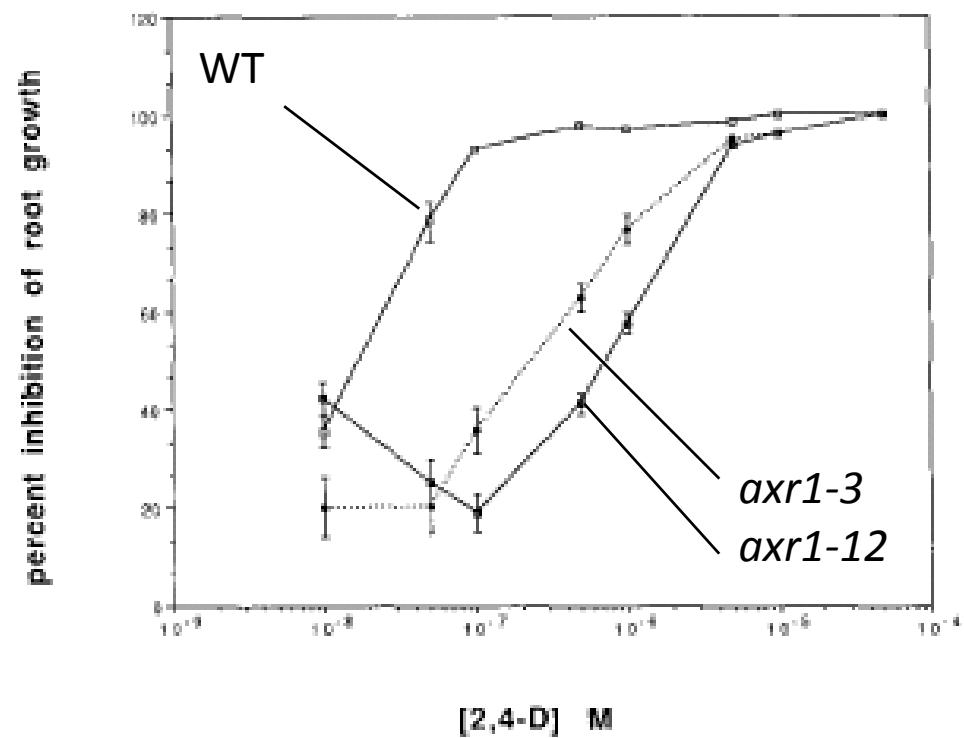


Auxin Response in der Wurzel:

2. Wurzelelongation auf Auxin

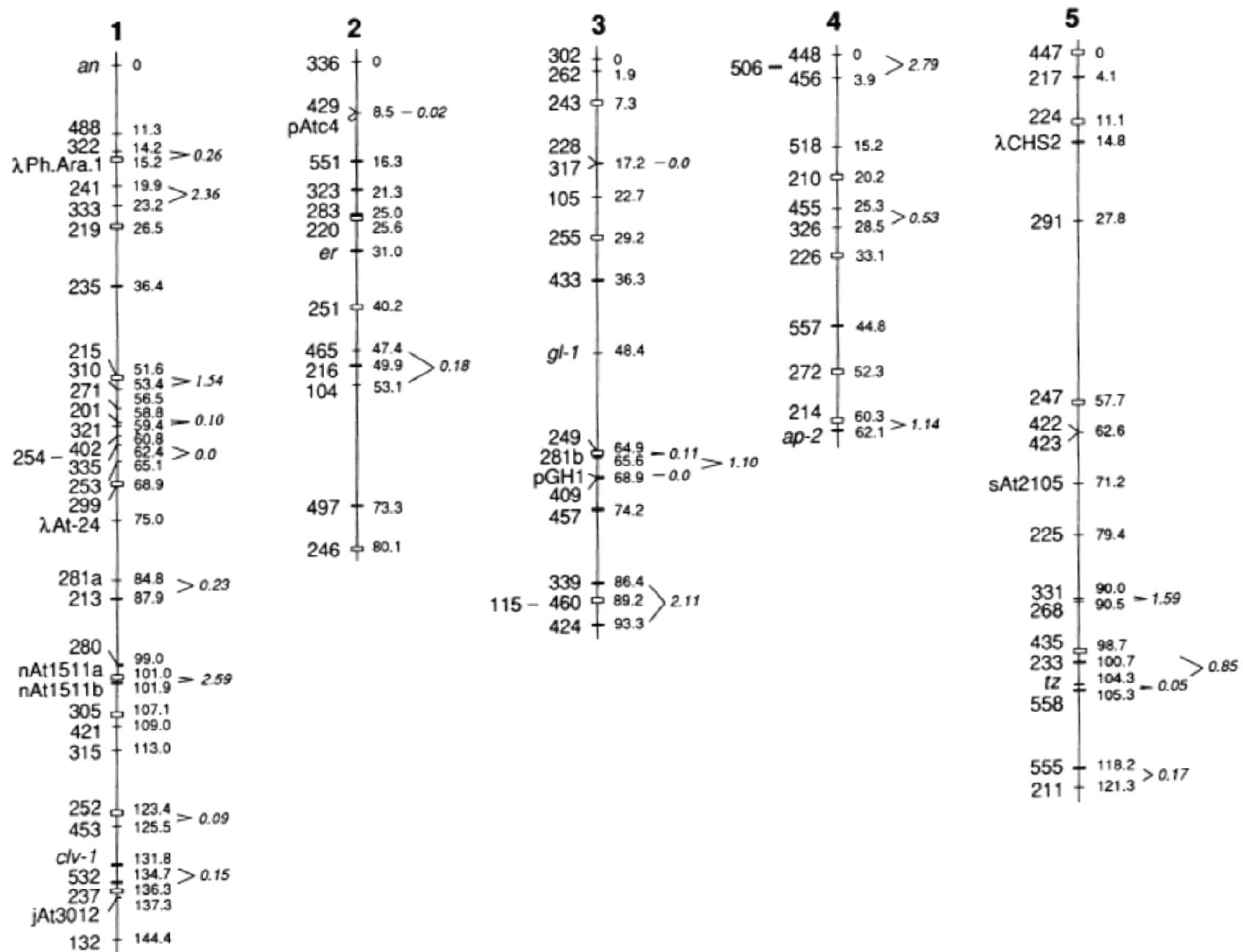


→ klassischer Auxin Response Defekt

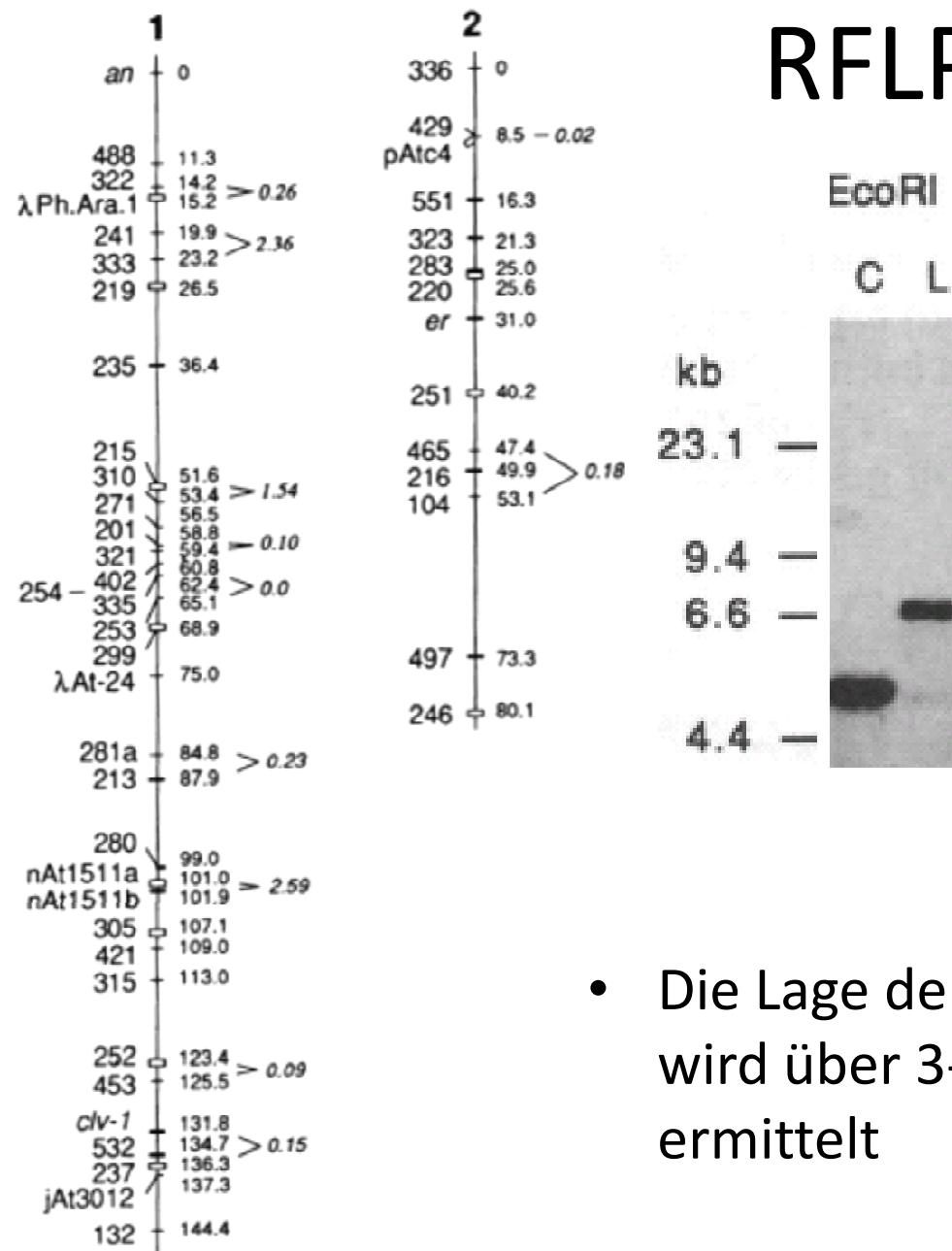


Kartierung des Gens: Map-based cloning

- Voraussetzungen:
 - Spaltende F2-Generation einer Kreuzung aus der Mutante (im Col-0 Hintergrund) mit einem anderen Genotyp (Ler)
 - Molekulare Marker zur Unterscheidung der 2 Genotypen (Col-0 und Ler) z.B. RFLP-Marker
 - genetische Karte der Markerpositionen



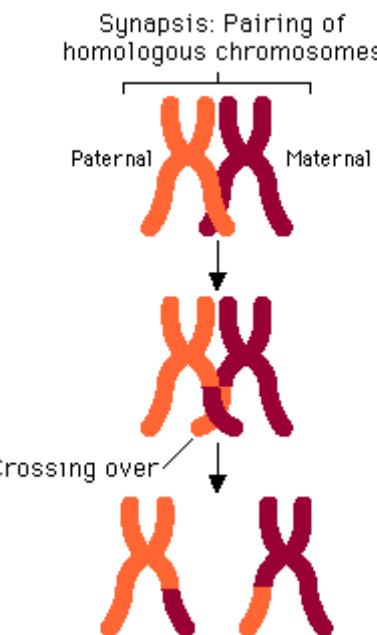
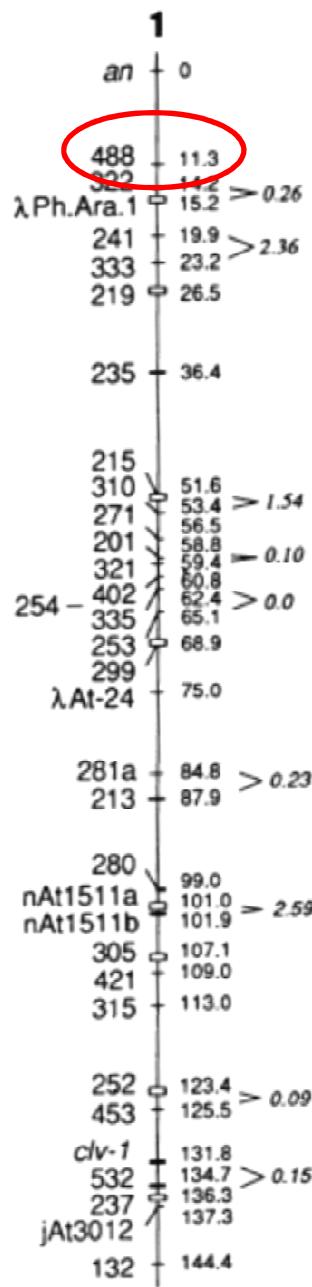
RFLP-Markerkarte



- An verschiedenen Positionen im Genom lassen sich Col-0 und Ler unterscheiden, da die DNA-Fragmente unterschiedlich groß sind (= molek. Marker)
- Die Lage der Marker zueinander wird über 3-Punkt Analysen ermittelt

Kartierung des Gens: Map-based cloning

- Voraussetzungen:
 - ✓ Spaltende F2-Generation einer Kreuzung aus der Mutante (im Col-0 Hintergrund) mit einem anderen Genotyp (Ler)
 - ✓ Molekulare Marker zur Unterscheidung der 2 Genotypen (Col-0 und Ler) z.B. RFLP-Marker
 - ✓ Karte der Marker
- Durchführung:
 - Untersucht die F2-Pflanzen auf:
 - *axr1* Phänotyp (= Auxinresistenz und Phänotypen)
 - Genotypen an verschiedenen Markerpositionen
 - Kopplungsanalyse:
 - tritt die *axr1* Mutation bevorzugt mit einem bestimmten Marker auf



je näher das Gen an einem bestimmten Marker liegt, desto weniger Rekombinationsereignisse treten auf (d.h. es liegt hauptsächlich der Col-0 und nicht der Ler Genotyp am Marker vor)

Table 6. Linkage Analysis between the AXR1 Gene and RFLP Markers on Chromosome 1

Markers	Recombination Frequency (%)	Number Scored ^a	χ^2 Associated ^b	P
RFLP 219	12.4 ± 2.0	136	123.3	<0.05 ^c
RFLP 253	47.8 ± 6.3	63	5.6	0.231
RFLP 488	2.6 ± 1.0	99	174.0	<0.05
phyA1	7.1 ± 2.0	102	135.0	<0.05

^a Refers to number of F3 families scored.

^b χ^2 associated is the total χ^2 adjusted for deviations of each individual marker from Mendelian segregation.

^c P value < 0.05 indicates deviation from nonlinkage (i.e., linkage).

Growth and Development of the *axr1* Mutants of *Arabidopsis*

Cynthia Lincoln, James H. Britton, and Mark Estelle¹

Department of Biology, Indiana University, Bloomington, Indiana 47405

Fazit:

axr1 Mutanten zeigen zahlreiche Phänotypen in verschiedenen Geweben (Spross und Wurzel!)

- AXR1 muss ein zentrales Element in der Vermittlung der Auxin response sein
 - auxin rezeptor oder wichtiges Element in der Signaltransduktion
- und/oder: eventuell einen Knotenpunkt darstellen, an dem Signale aus verschiedenen Hormon-Signalwegen zusammenlaufen

Kartierung des betroffenen Gens (*AXR1*) ergab eine Lage auf Chromosom 1

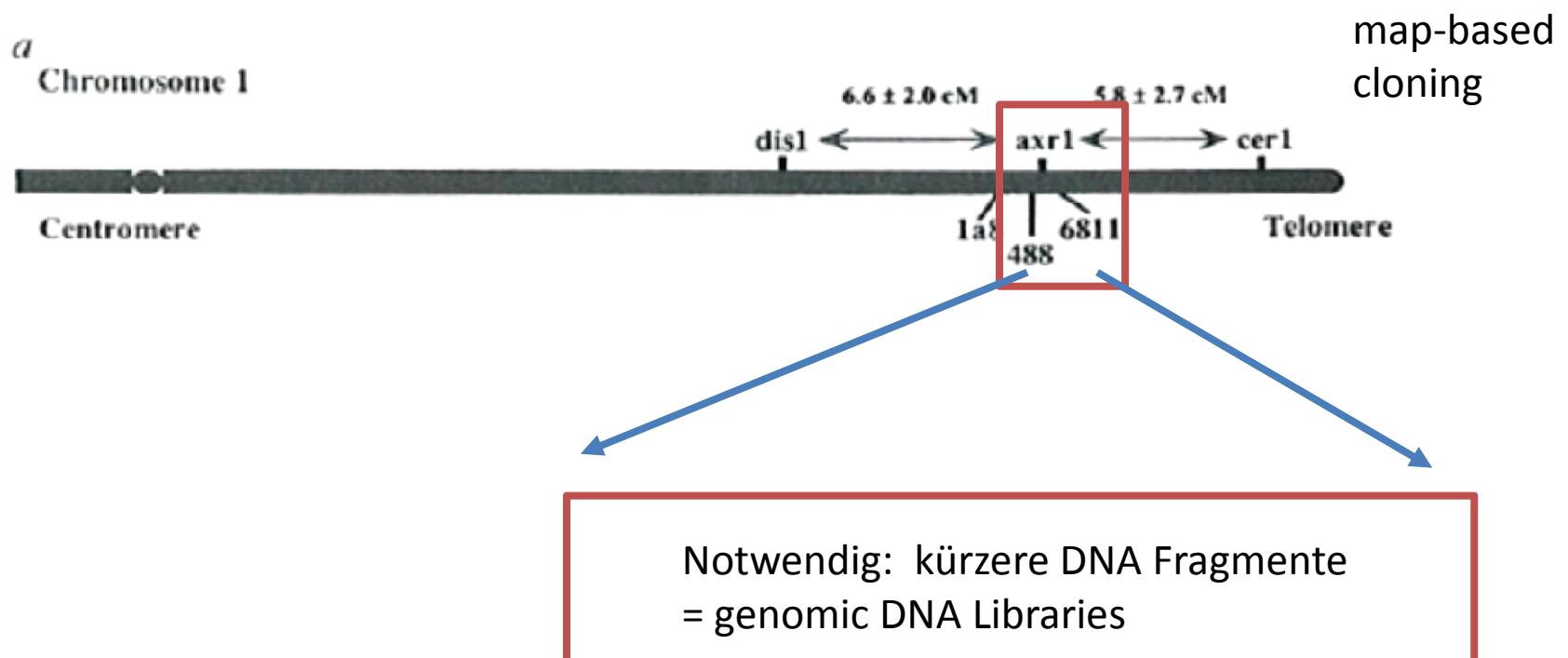
***Arabidopsis* auxin-resistance gene *AXR1* encodes a protein related to ubiquitin-activating enzyme E1**

**H. M. Ottoline Leyser, Cynthia A. Lincoln*,
Candace Timpte, Douglas Lammer,
Jocelyn Turner & Mark Estelle†**

Department of Biology, Indiana University, Bloomington,
Indiana 47405, USA

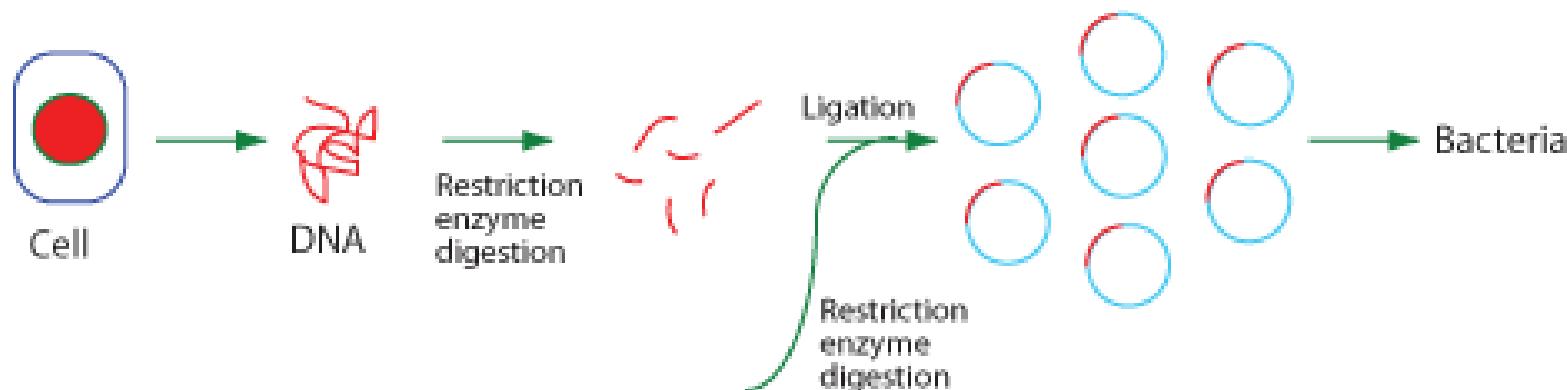
Ziel: Kartierung /Identifizierung des *AXR1* Gens

chromosome walk

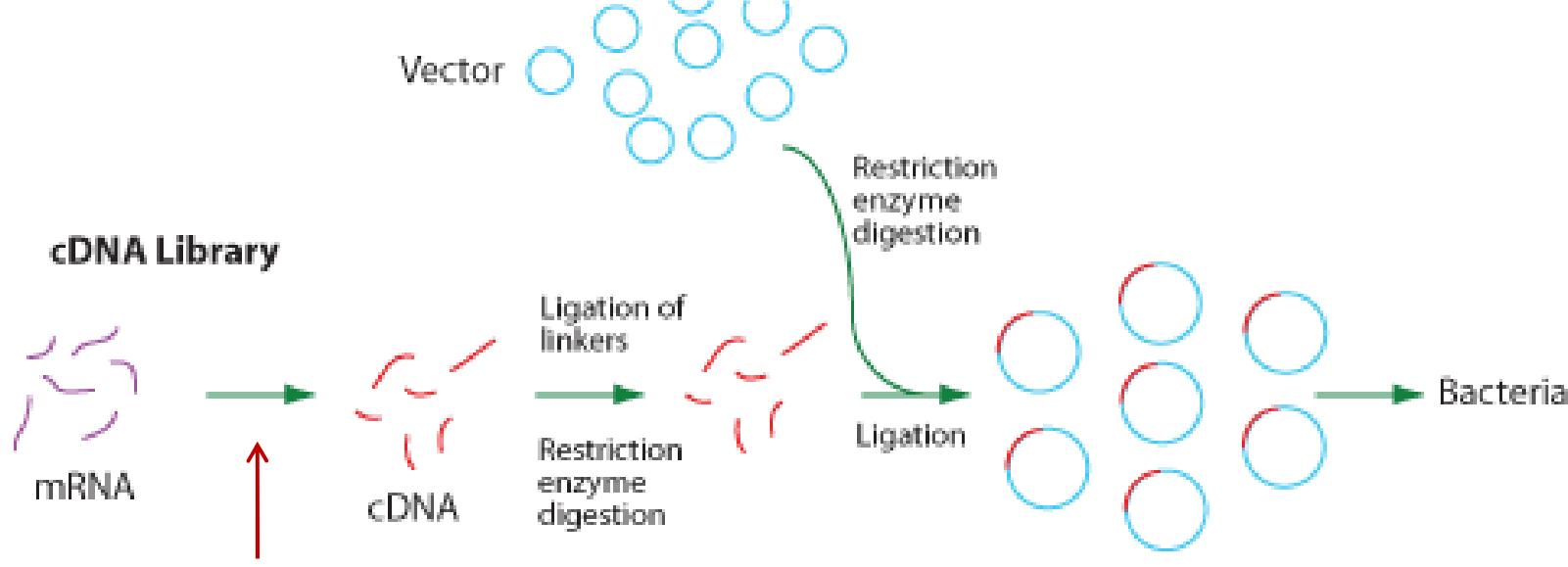


DNA libraries

Genomic Library



cDNA Library

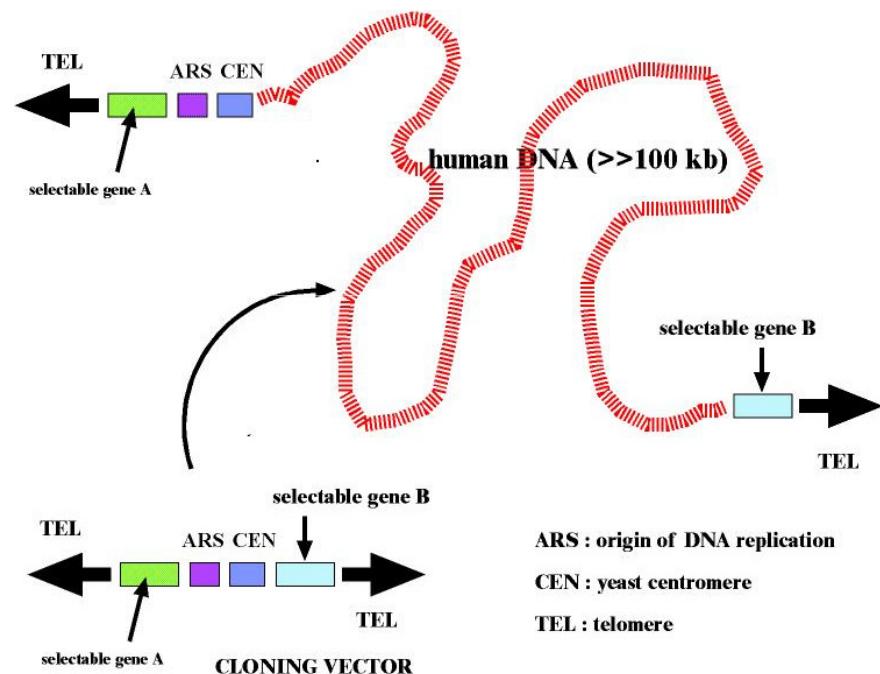


Reverse Transcription

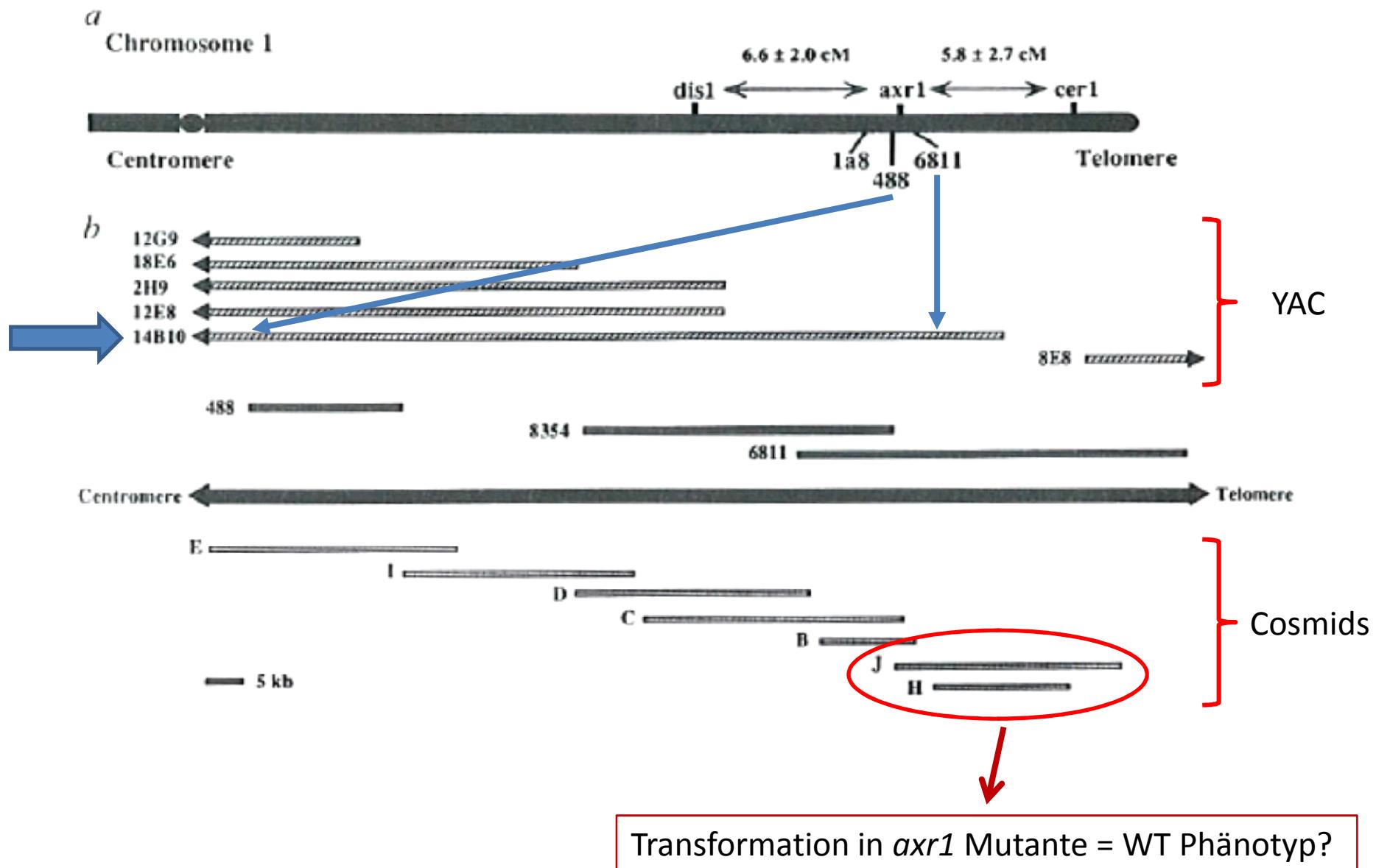
YACs, cosmids.....

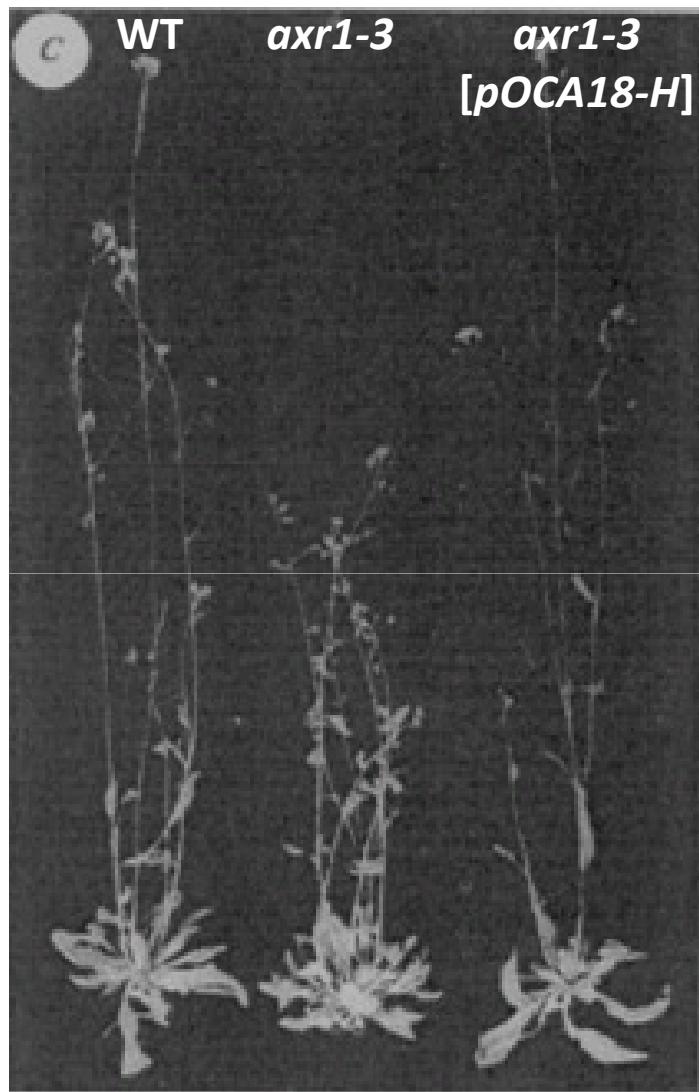
Approximate maximum length of DNA that can be cloned into vectors

Vector type	Cloned DNA (kb)
<u>Plasmid</u>	20
<u>lambda phage</u>	25
<u>Cosmid</u>	45
<u>BAC</u> (bacterial artificial chromosome)	300
<u>YAC</u> (yeast artificial chromosome)	1000



AXR1 - Chromosome Walking





Cosmid H kann den Phänotyp der
axr1-3 Mutante aufheben

→ Cosmid pOCA18-H enthält das *AXR1* Gen

→ Cosmid pOCA18-H enthält das *AXR1* Gen



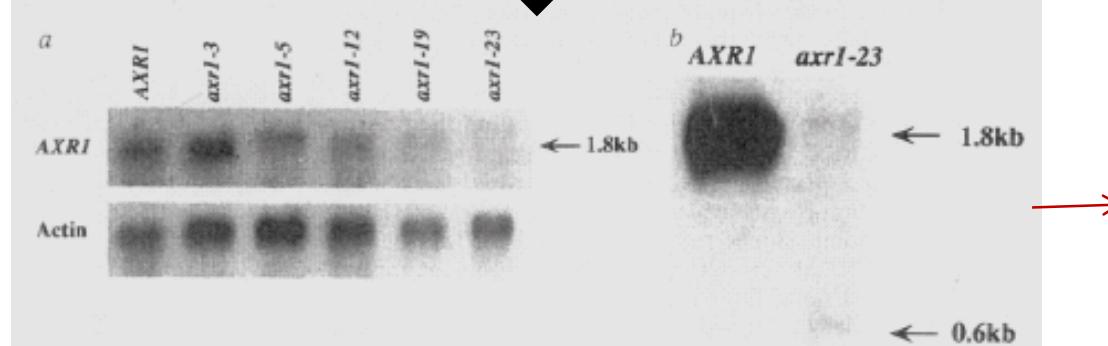
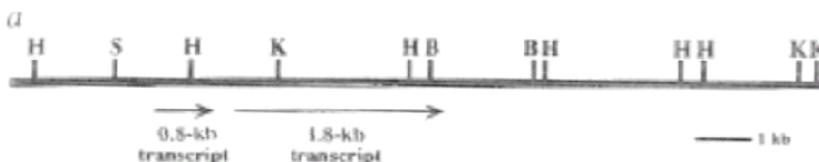
cDNA Bibliothek Screen mit pOCA18-H



2 cDNAs identifiziert → 0.8 und 1.8 kb

→ welche cDNA Klone sind homolog zu pOCA18-H

→ potentielle *AXR1* cDNAs



Northern Blot:
Wieviel Transkript (1,8 kb)
liegt in den *axr1* Mutanten
vor?

1.8 kb cDNA abwesend in *axr1-23*!

Sequenzierung des potentiellen AXR1 Gens

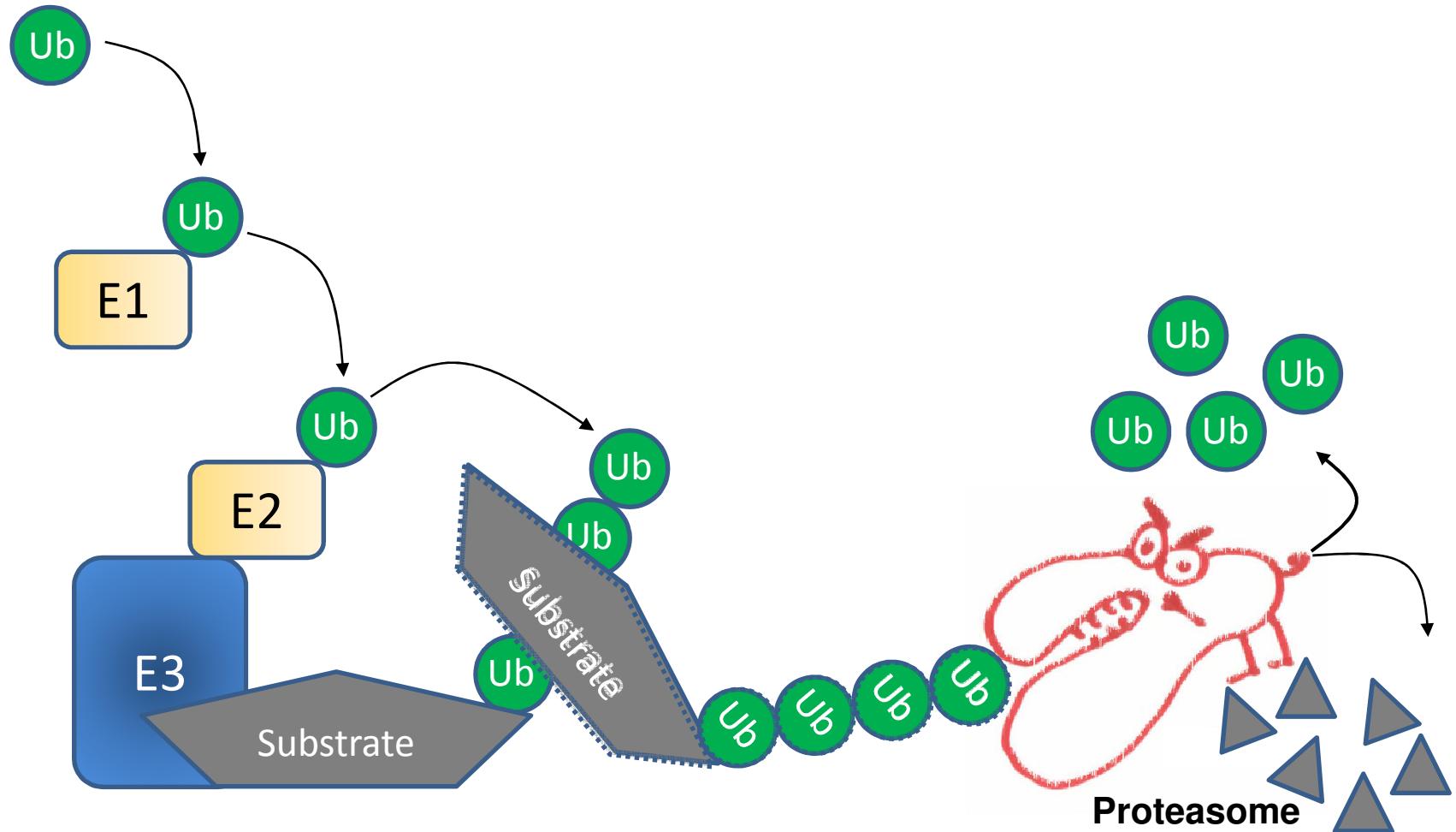
AXR1 Protein: 540 AS, ca. 60 kD

human	M S S S P L S K K R	R V S G P D P K P G	S N C S P A Q S V L	S E V P S V P T N G	M A K N G S E A D I	D E G L Y S R Q L Y	60
yeast M S S N N S G L	S	A A G E I	D E S L Y S R Q L Y
wheat	.	. M L P R K R	E I V A G E V E D L	. Q K K T R A G E G .	. E V T R E E G D	A A M A G R G N E I	D E D L H S R Q L A
AXR1 M Q A V K R S R R H V	. E E P T M V E	P K T K Y D R Q L R
human	V L G H E A M K R L	Q T S S V L V S G L	R G L G V E I A K N	I I L G G V K A V T	L H D Q G T A Q W A	D L S S Q F Y L R E	120
yeast	V L G K E A M L K M	Q T S N V L I L G L	K G L G V E I A K N	V V L A G V K S M T	V F D P E P V Q L A	D L S T Q F F L T E	84
wheat	V Y G R E T M K R L	F G S N V L V S G L	O G L G A E I A K N	L V L A G V K G V T	L H D D G N V E L W	D L S S N F F L S E	113
AXR1	I W G E V G Q A A L	E E A S I C L L N C	G P T G S E A L K N	L V L G G V G S I T	V V D G S K V Q F G	D L G N N F M V D A	89
human	E D I G K N R A E V	S Q P R L A E L N S	Y V P V T A Y T G P	L V E	D F L S G F Q V V V L	T N T . P L E D Q L	173
yeast	K D I G Q K R G D V	T R A K L A E L N A	Y V P V N V L D S L	D D V	T Q L S Q F Q V V V A	T D T V S L E D K V	138
wheat	N D V G Q N R A Q A	C V O K L Q E L N N	A V L V S A L T G D	L T K	E H L S K F Q A V V F	T D . I S L D K A J	166
AXR1	K S V G Q S K A K S	V C A F L Q E L N D	S V N A K F I E E N	P D T L I T T N P S	F F S Q F T L V I A	T Q L V E . D S M L	148
human	R V G E F C H N . .	R G I K L V V A D T	R G L F G Q L F C D F G E E	M I L T D S N G E Q	P L S A M V S M V T	225
yeast	K I N E F C H S . .	S G I R F I S S E T	R G L F G N T F V D L G D E	F T V L D P T G E E	P R T G M V S D I E	190
wheat	E F D D . H S Q Q	P P I A F I S S E V	R G L F G S V F C D F G P E	F T V L D D V D G E E	P H T G I V A S I S	220
AXR1	K L D R I C H I D A N	V K L V L V R S Y G	LAG F V R I S V K	E H P I I D S K P D	H F L D D L R L N N	P W P E L K S F V E	208
human	K D N P G V V T C L D E A R H	G F E S G D F V S	249
yeast	P D . . G T V T M L D D N R H	G L E D G N F V R	212
wheat	N D N P A L V S C V D D E R L	E F Q D G D L V V	244
AXR1	T I D L N V S E P A	A A H K H I P Y V V	I L V K M A E E W A	Q S H S G N L P S T	R E E K K E F K D L	V K S K M V S T D E	268
human	F S E V G G M V E	L N G N G P M E I K V L G	271	
yeast	F S E V E G L D K	L N D G T L F K V E V L G	244	
wheat	F S E V H G M T E	L N D G K P R K V K N A R	266	
AXR1	D N Y K E A I E A A	F K V F A P R G I S	S E V O K L I N D S	C A E V N S N S S A	F W V M V A A L K E	F V L N E G G G E A	328
human	P Y T F S I . C D T	S N F S D V I	294
yeast	P F A F R I . G S V	KEY G E Y K	267
wheat	P Y S F F L E E D T	S S F G A Y V	290
AXR1	P L E G S I P D M T	S S T E H Y I N L Q	K I Y L A K A E A D	F L V I E E R V K N	I L K K I G R D P S	S I P K P T I K S F	388
human	V K V P K K I S F K S L V A S L A	E P . D F V V T D F	. . . A K F S R P A	Q L H I G F Q A L H	Q F C A Q H . G R P	346
yeast	V K V P R K I S F K S L K Q Q L S	N P . E F V F S D F	. . . A K F D R A A	Q L H L G F O A L H	Q F A V R H N G E L	320
wheat	V K P P K V I K F K P L K E A M S	E P G E F L M S D F	. . . S K F E R P P	L L H L A F O A L D	K F R T . E L S R F	343
AXR1	C K N A R K L K L C	R Y R M V E D E F R	N P S V T E I Q K Y	L A D E D Y S G A M	G F Y I I L L R A A D	R F A A . H Y N K F	447
human	P R P R N E E D A A	E L V A L A Q A V N	A R A L P A V Q Q N	. N L D E D L I R K	L A Y V A A G D L A	P I N A F I G G L A	405
yeast	P R T M N D E D A N	E L I K L V T D L S	V Q Q P E V L G E G	V D V N E D L I K E	L S Y Q A R G D I P	G V V A F F G G L V	380
wheat	P V A G S T D D V Q	R V I E Y . . A I S	I N D T L G D R K L	E E I D K K L L H H	F A S G S R A Y V L N	P M A A M F G G I V	401
AXR1	P G Q F D G G M D E	D I S R L K T T A L	S L L T D L G C N G	S V L P D D L I H E	M C R F G A S E I H	V V S A F V G G I A	507
human	A Q E V M K A C S G	K F M P I M Q W L Y	F D A L E C L P E D	K E V L T E D K C L	Q R Q N R	450
yeast	A Q E V L K A C S G	K F T P L K Q F M Y	F D S L E S L P D P	K N F P R N E K T T	Q P V N S R Y D N Q	I A V F G L D F Q K	440
wheat	G Q E V V K A C S G	K F H P L Y Q F F Y	F D S V E S L P V D	. . . P L E P G D L	K P K N S R Y D A Q	I S	450
AXR1	S Q E V I K L V T K	Q F V P M L G T Y I	F N G I D H K S Q L	L K L	540

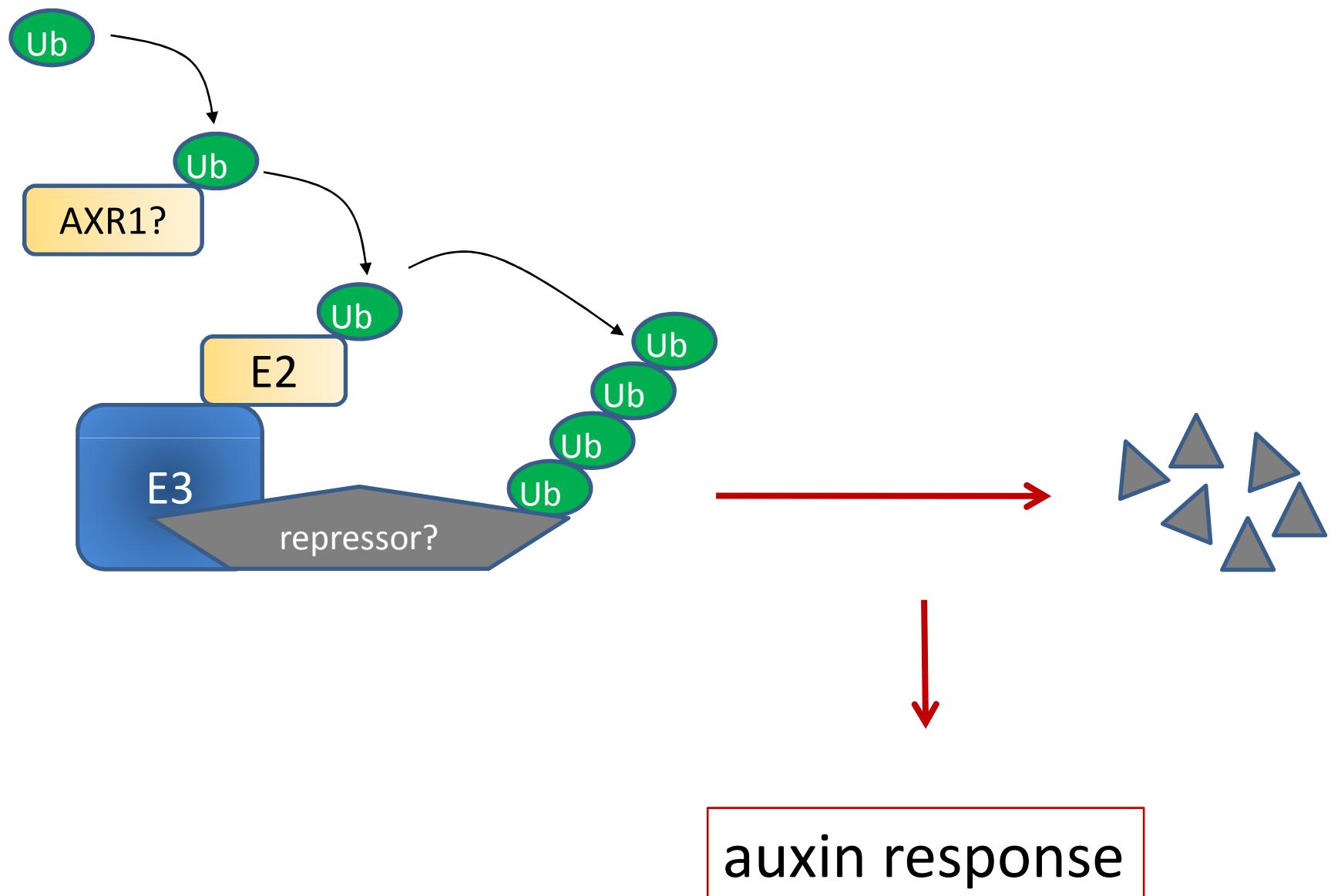
axr1-3
C → Y

- similarity to ubiquitin-activating enzyme E1
- *axr1-3* mutation alters conserved C- residue
- ubiquitin-proteasome pathway

The ubiquitin system



preliminary model



Termine:

datum	autoren	titel	link	wer stellt vor?
22.04.2009	lincoln et al. (1990)	Growth and development of the <i>axr1</i> mutants of <i>Arabidopsis</i>	Plant Cell 2:1071 full text pdf + glossar	
	leyser et al. (1993)	<i>Arabidopsis auxin-resistance gene AXR1 encodes a protein related to ubiquitin-activating enzyme E1</i>	Nature 364:161 abstract + glossar	carolin → ppt.pdf
29.04.2009	Ulmasov et al. (1997)	ARF1, a transcription factor that binds to auxin response elements.	Science 276:1865	
	Ballas et al. (1993)	Identification of the Auxin-responsive Element, AuxRE, in the Primary indoleacetic Acid-inducible Gene, PS-IAA4/5, of Pea (<i>Pisum sativum</i>)	J Mol Biol 233:580	carolin
06.05.2009	Ulmasov et al. (1999)	Dimerization and DNA binding of auxin response factors	Plant J 19:309	x
13.05.2009	Ruegger et al. (1998)	The TIR1 protein of <i>Arabidopsis</i> functions in auxin response and is related to human SKP2 and yeast Grr1p	Genes & Development 12:198 full text pdf + glossar	x
20.05.2009	Gray et al. (1999)	Identification of an SCF ubiquitin-ligase complex required for auxin response in <i>Arabidopsis thaliana</i>	Genes & Development 13:1678 full text pdf + glossar	x
27.05.2009	Gray et al. (2001)	Auxin regulates SCFTIR1-dependent degradation of AUX/IAA proteins	Nature 414:271 abstract + glossar	x
03.06.2009	Dharmasiri et al. (2003)	Auxin Action in a Cell-Free System	Current Biology 13:1418 abstract + glossar	x
10.06.2009	Kepinski and Leyser (2005)	The <i>Arabidopsis</i> F-box protein TIR1 is an auxin receptor	Nature 435:446 abstract + glossar	x
	Callis (2005)	news and views zu den beiden papers (= kommentar)	news and views	
17.06.2009	Tan et al. (2007)	Mechanism of auxin perception by the TIR1 ubiquitin ligase	Nature 446:640 abstract + glossar	x
	Guilfoyle (2007)	news and views zum paper (= kommentar)	news and views	
24.06.2009	Hayashi et al. (2008)	Small-molecule agonists and antagonists of F-box protein–substrate interactions in auxin perception and signaling	PNAS 105:5632	x