The role of *p19* gene in the transformation efficiency of amorphadiene synthase in Artemisia annua for artemisinin production

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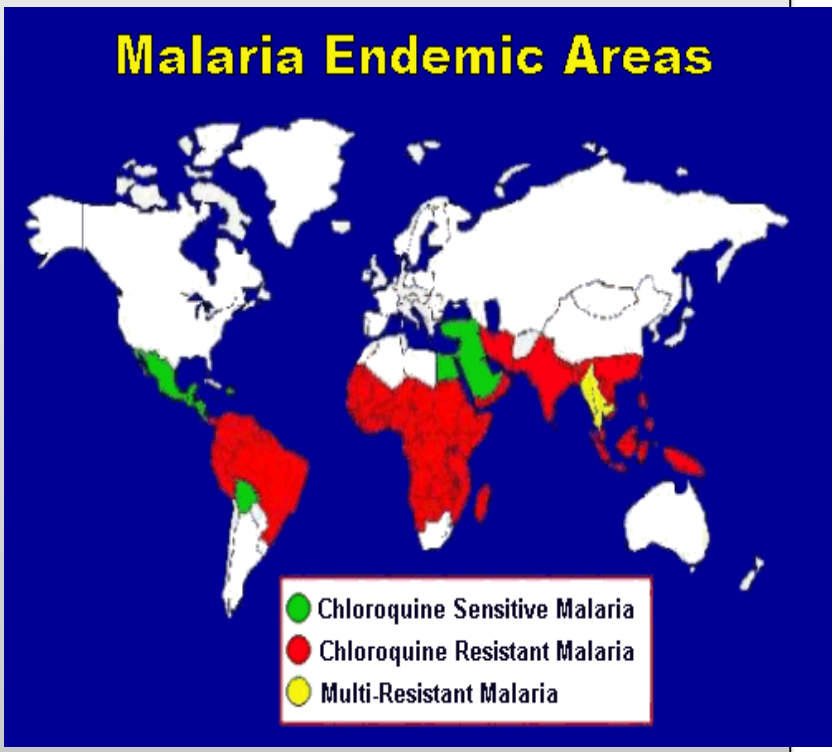
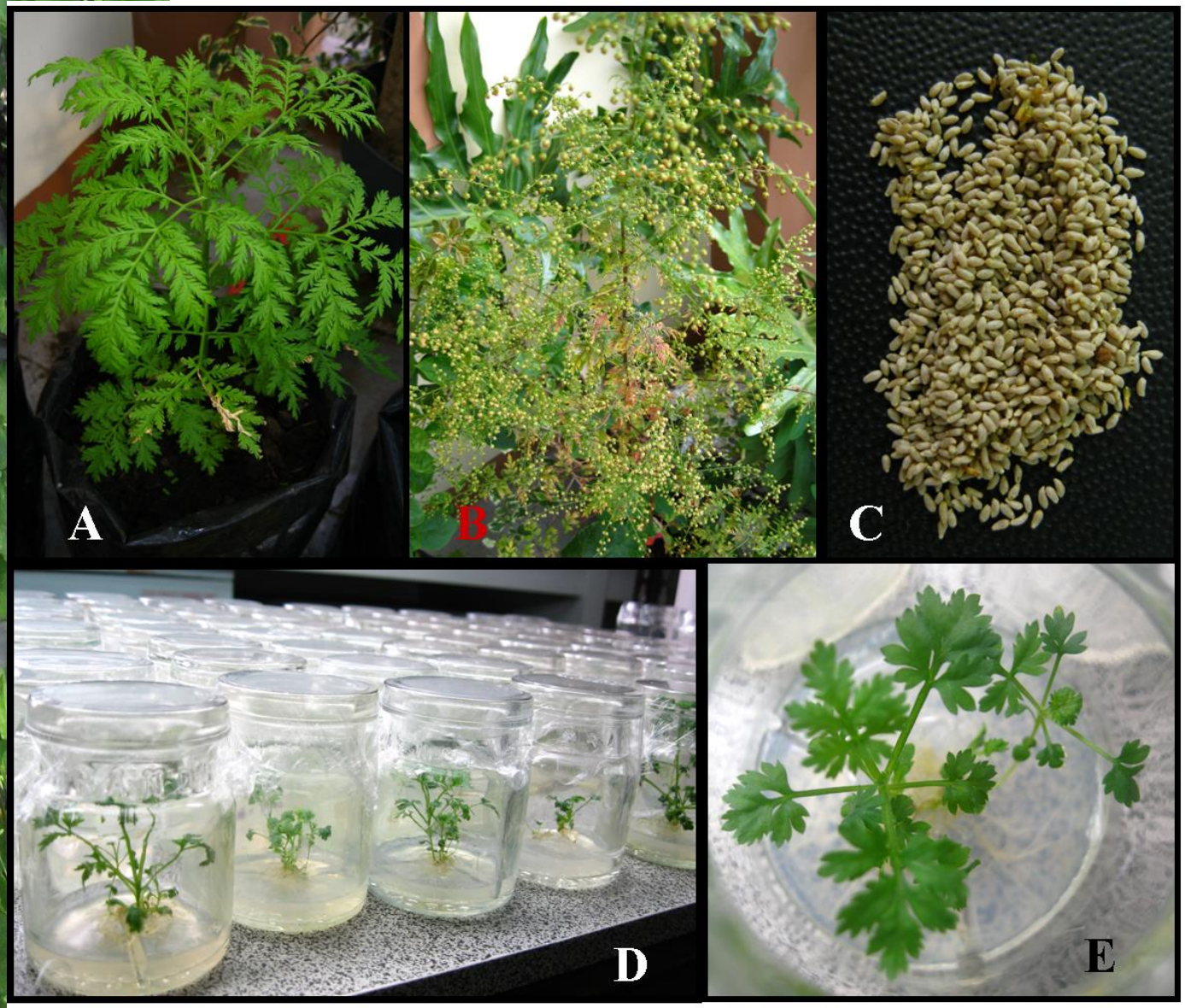
INTRODUCTION

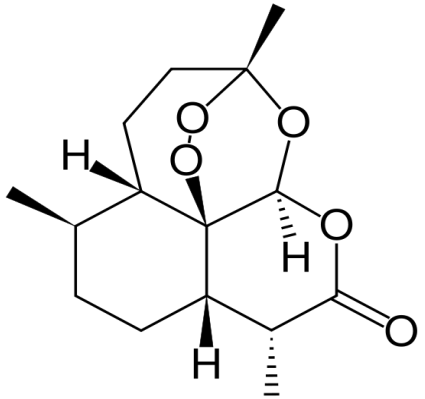
- Malaria: widely spread in the world → TROPICAL AREA
- WHO(2005) : 300-500 billion infected every year, more than 1 billion died
- *Plasmodium falciparum* → resistance for several antimalarial drugs
- WHO : Artemisinin Based Combination Therapies (ACT's)



www.nhrc-qa.org/en/news.php%3Fitem.179.4

INTRODUCTION





artemisinin from
Artemisia annua

Introduction

- Production of secondary metabolites from plants is usually poor

  ex. Vincristine and vinblastine from *Catharanthus roseus*

  Podophyllotoxin from *Podophyllum spp, Linum spp*

  Artemisinin from Artemisia annua

- Biotechnology is a method that can be used to improve the production of secondary metabolites from plants

  ex. Production of podophyllotoxin from cell suspension of *Linum flavum* L, *Podophyllum hexandrum*

  Production of indole alkaloids from transgenic plants

Plant Biotechnology methods applied

- *Regulation of secondary metabolites using plant cell culture*

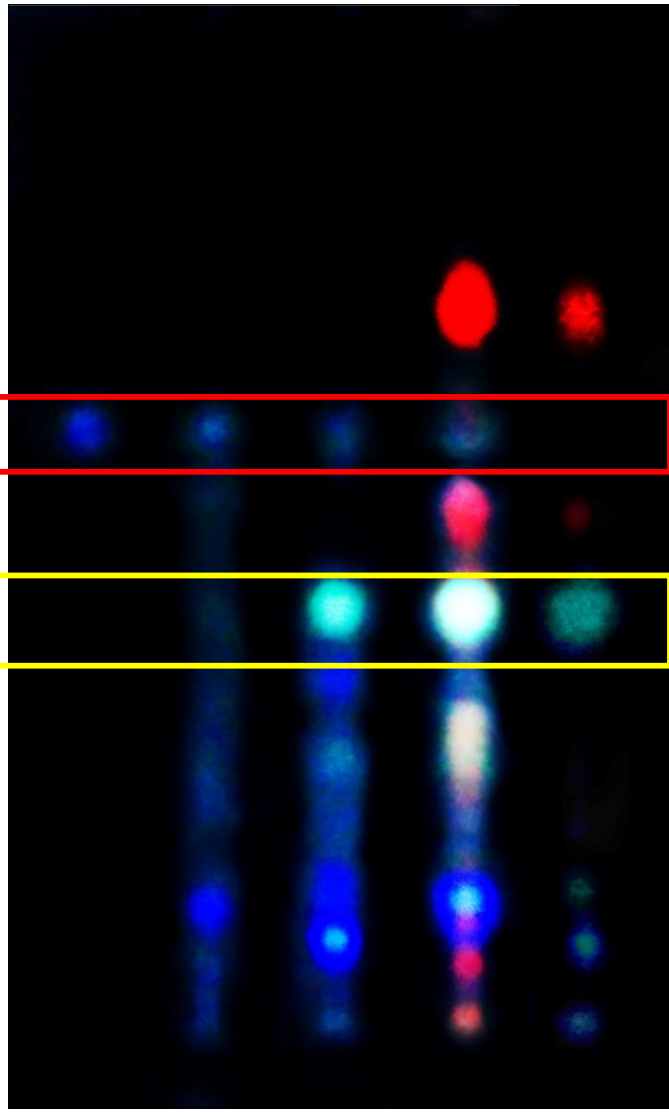
  Feeding, Elicitation, biotransformation, etc

- *Agrobacterium-transformed mediated transformation*

  Agrobacterium rhizogenes (Hairy roots culture), Agrobacterium tumefaciens

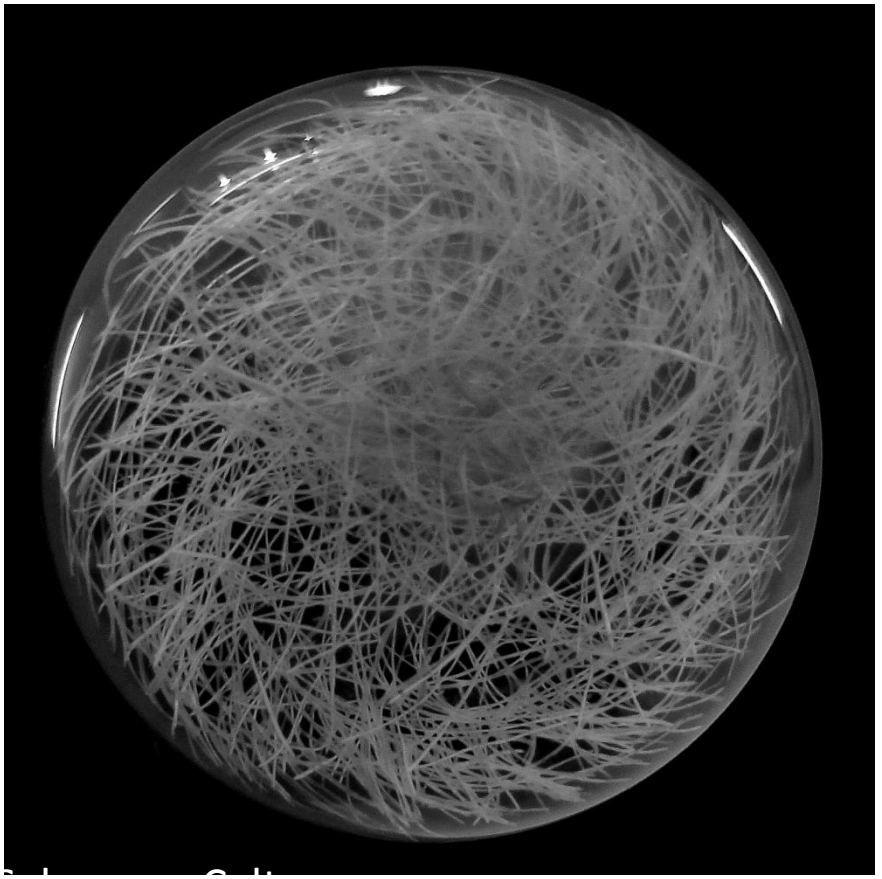
- *Combinatorial Biosynthesis*

TLC & Artemisinin Content

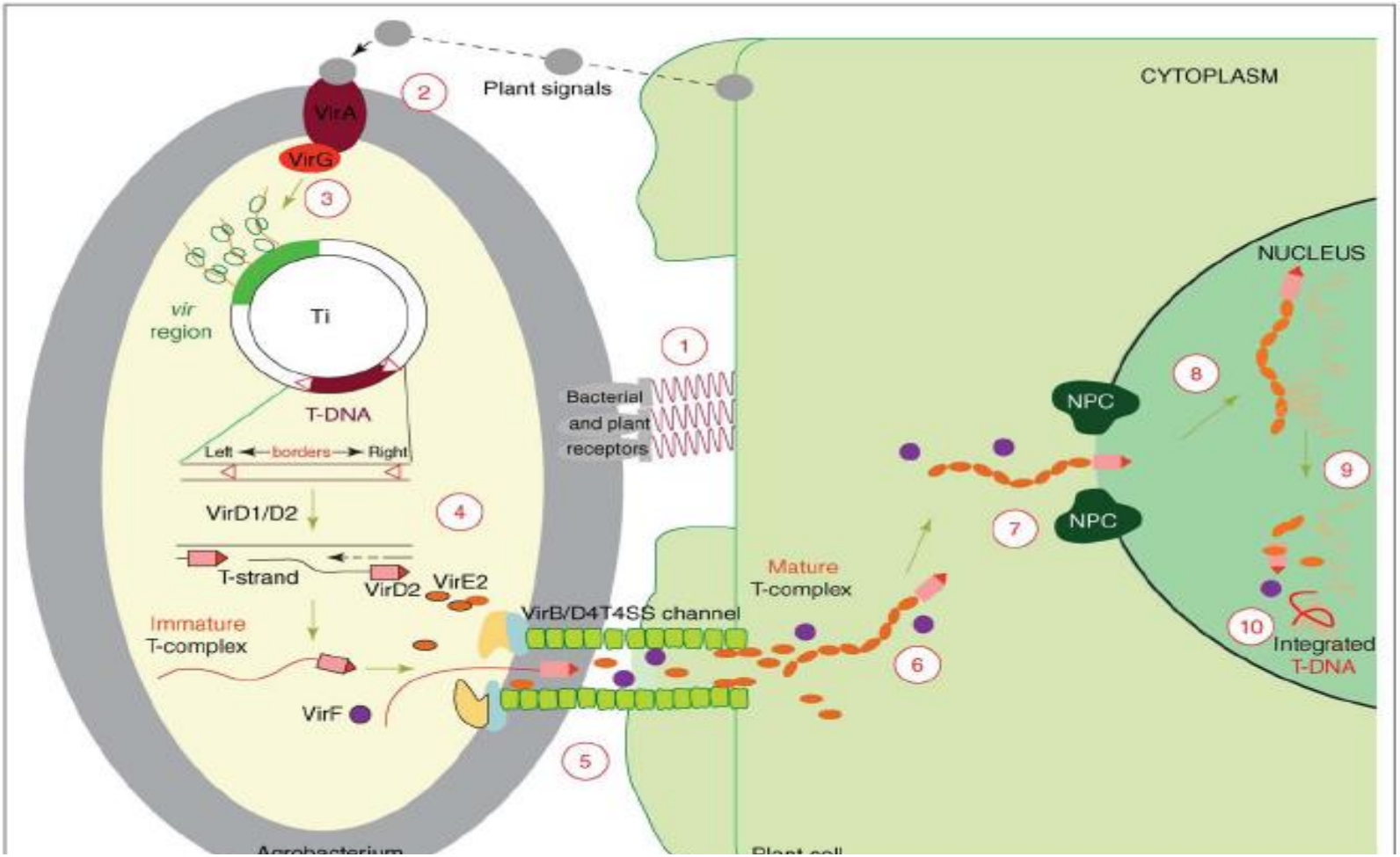


Spot	[Artemisinin], %
AN : Artemisinin	1,6 ug
SD : Wild leaf	0,36% ± 0,029
HR : Hairy root	0,29% ± 0,025
IL : in vitro leaf	0,16% ± 0,019
Untransformed root	0%

Agrobacterium rhizogenes-mediated transformation

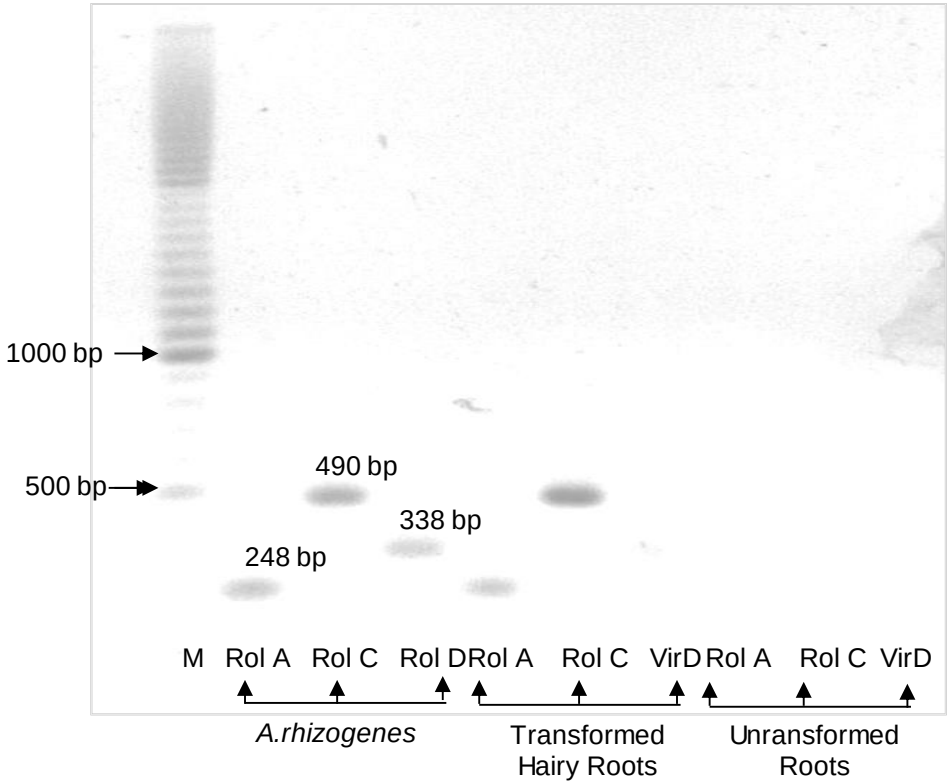


- ***Induction the hairy root culture using Agrobacterium rhizogenes***



A model for the *Agrobacterium*-mediated genetic transformation. The transformation process comprises 10 major steps and begins with recognition and attachment of the *Agrobacterium* to the host cells (1) and the sensing of specific plant signals by the *Agrobacterium* VirA/VirG two-component signal-transduction system (2). Following activation of the *vir* gene region (3), a mobile copy of the T-DNA is generated by the VirD1/D2 protein complex (4) and delivered as a VirD2–DNA complex (immature T-complex), together with several other Vir proteins, into the host-cell cytoplasm (5). Following the association of VirE2 with the T-strand, the mature T-complex forms, travels through the host-cell cytoplasm (6) and is actively imported into the host-cell nucleus (7). Once inside the nucleus, the T-DNA is recruited to the point of integration (8), stripped of its escorting proteins (9) and integrated into the host genome (10). A detailed model of the host cellular mechanisms and the role of plant-specific factors in the transformation process are given in Figure 2. (This illustration was reproduced, with modifications, from [28•] with permission.).

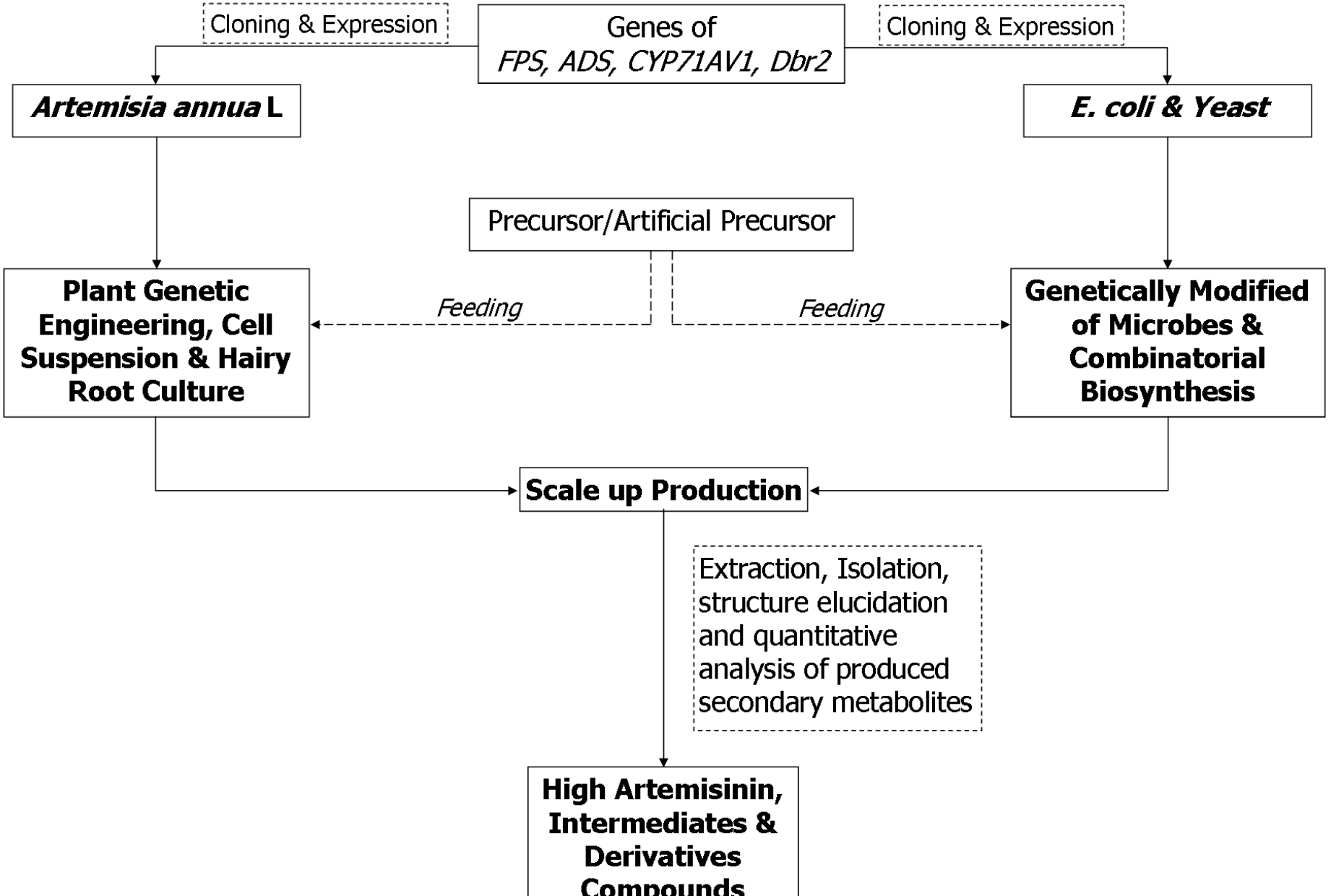
- ***Control of genetic transformation***

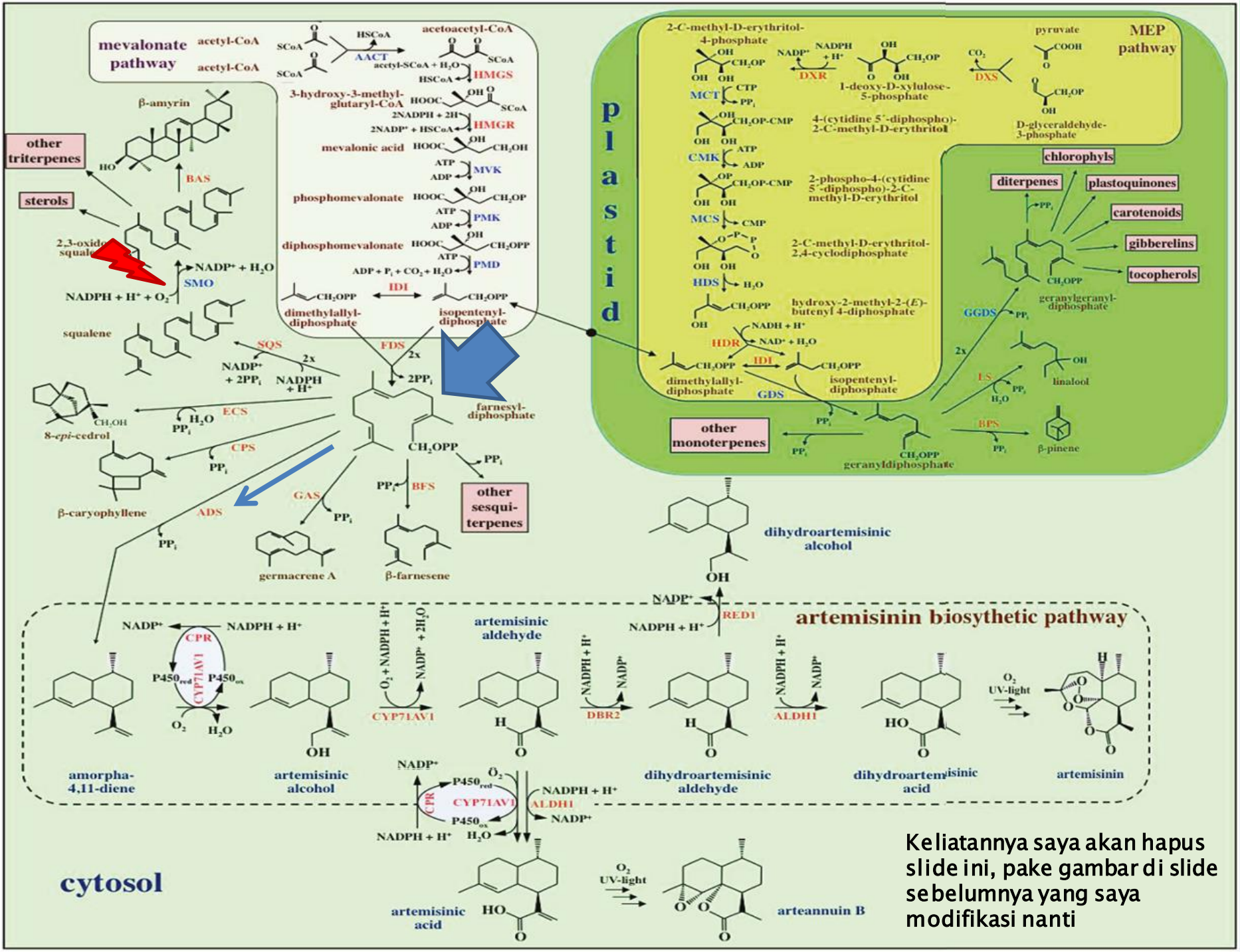


Gel electroferesis of PCR product from *Agrobacterium rhizogenes*, transformed hairy root and untransformed root of *Centella asiatica*

Ruslan KR, **Elfahmi**, Selfitri, Pharmacognosy Magazine, in press

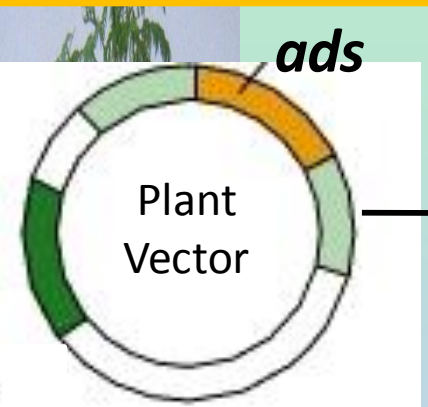
Roadmap of our genetic engineering research on artemisinin production in *Artemisia annua*

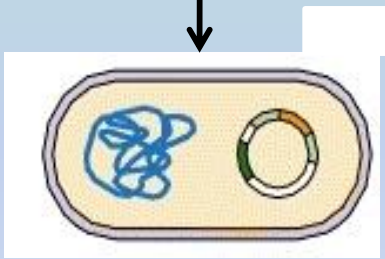




Keliatannya saya akan hapus slide ini, pake gambar di slide sebelumnya yang saya modifikasi nanti

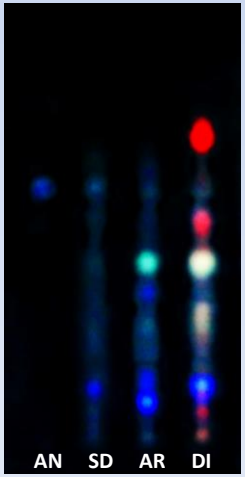
Strategy for Enhancement of Artemisinin via Overexpression of *ADS*





Agrobacterium

Leaf of *A. annua*



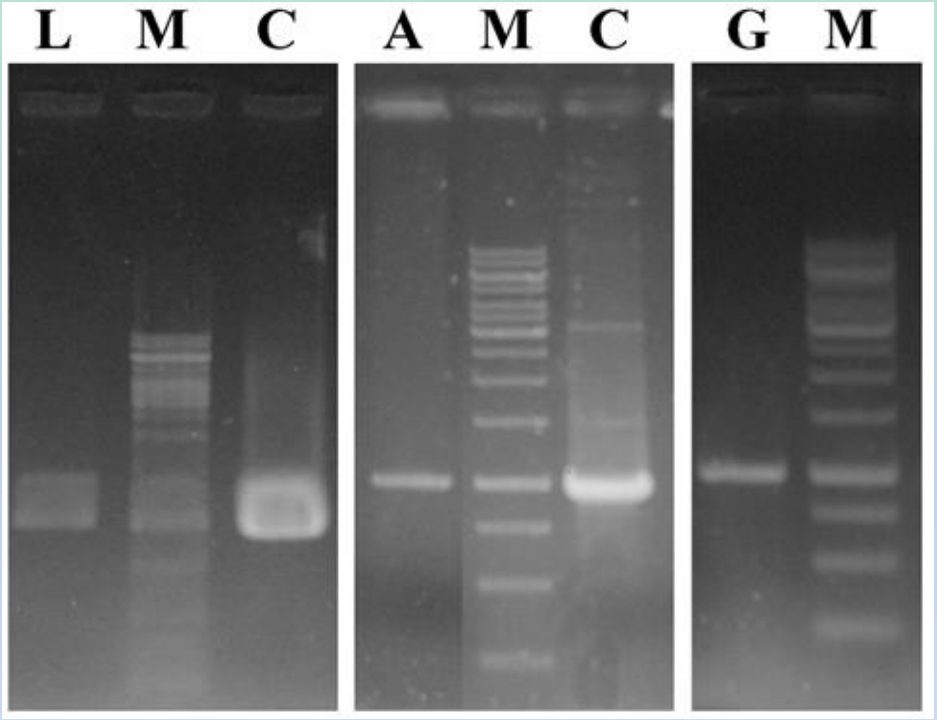
High Artemisinin

Cell Suspension

Hairy/Adventitious Roots

Transgenic Plant

Transformation efficiency of *Agrobacterium tumefaciens* into *Artemisia annua* L. via



PCR analysis of pCAMBIA 1303 from transformant of *A. tumefaciens* strains: LBA4404 (L), AGl1 (A), GV3101 (G), Marker 1 kb (M) and pCAMBIA 1303 as a *gus*-positive control (C)

Elfahmi et al. Pharmacognosy magazine 2014

Genetic transformation of *Artemisia annua* L. using *Agrobacterium tumefaciens*

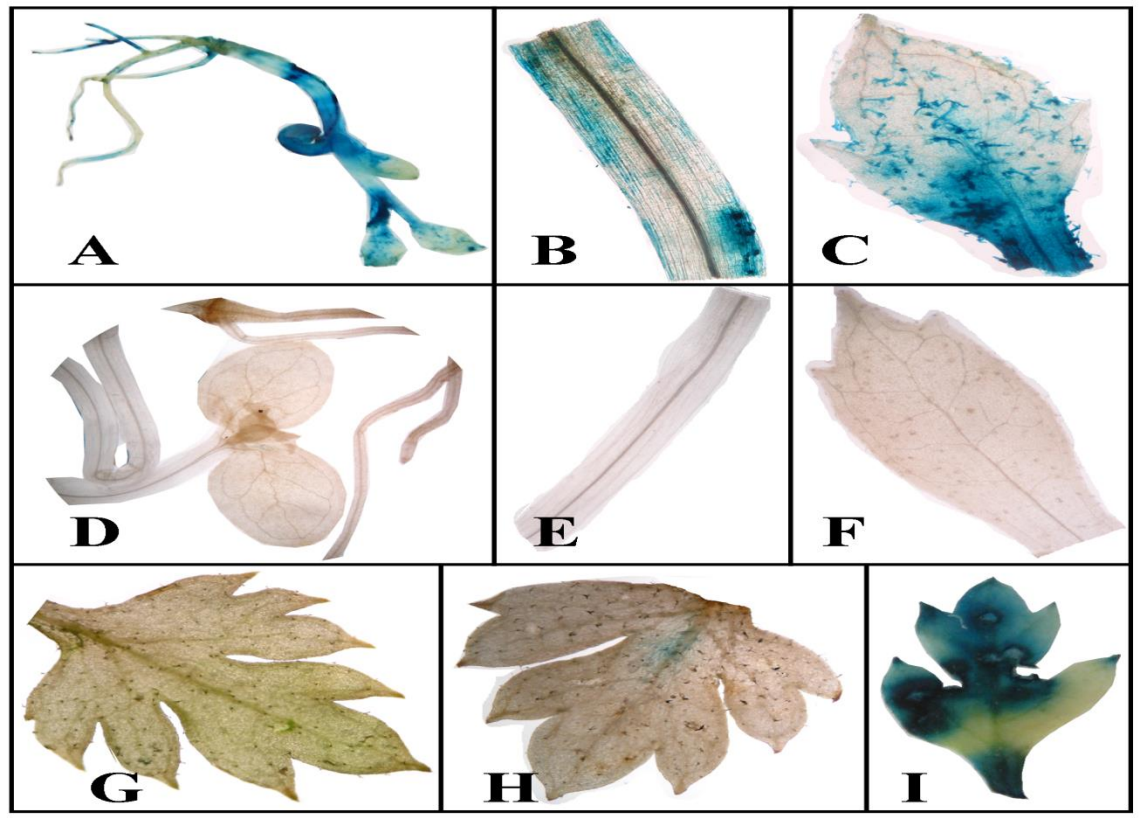
Strain	Area mean of GUS expression (%) [a]	Infection frequency (%) [b]
LBA4404	57% ±0,3 (*n=20*)	45,45% (*n=44*)
GV3101		49,25% (*n=67*)
AGl1		70,91% (*n=55*)

Infection frequency and area of GUS spots of *A.annua* after co-cultivation with three *A. tumefaciens* strains harboring the binary vector pCAMBIA 1303

a : *n* is total of GUS positif-leaves

b : *n* is total of leaves explant

Genetic transformation of *Artemisia annua* L. using *Agrobacterium tumefaciens*



Histochemical GUS assays of the transformed leaf of *A.annua*: Explant from 2-weeks-old seedling (A-F) and from 2-month-old-seedling (G-I). Transformed explant (A-C), whole explant (A), hipocotyl (B), and leaf (C). Untransformed explants (D-G). Leaf without wounds (H) and wounded leaf (I)

GUS analysis of ads transformed *Artemisia annua* using *Agrobacterium tumefaciens*

Explants (leaves)

Cleaned, dried

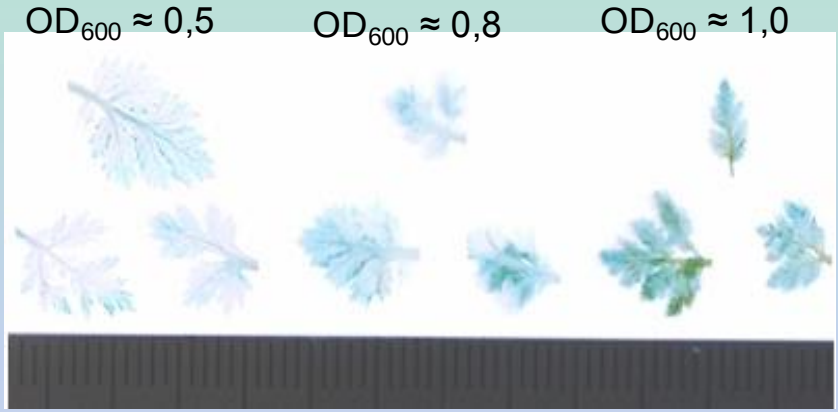
Incubated in staining x-gluc, 24 jam, 37ºC

Put in alcohol solution

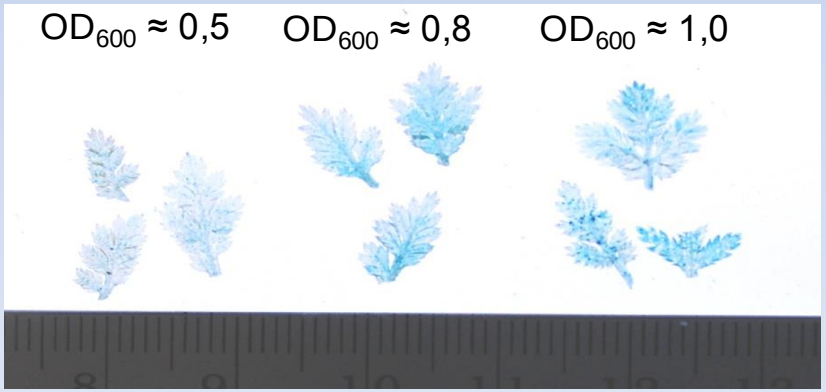
Blue color for transformed part

Analysis of blue area

- Treatment 1

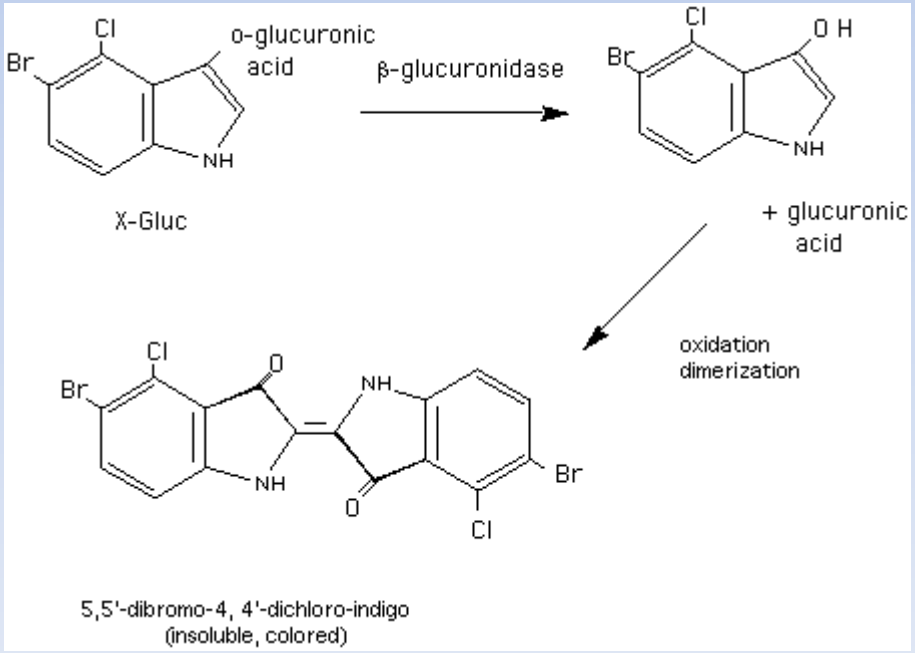


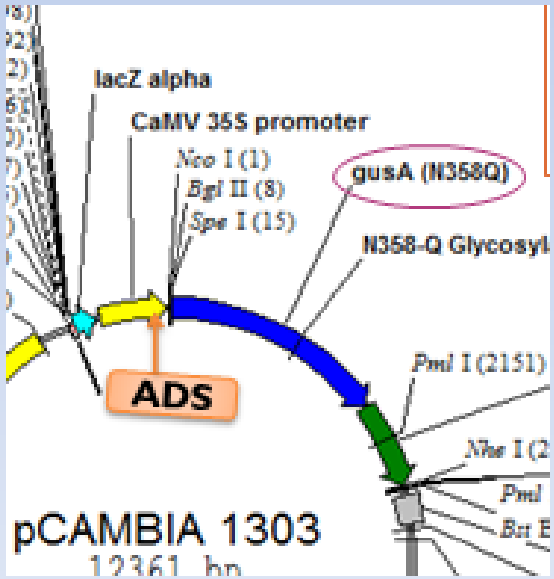
- Treatment 2



Expression analysis of GUS transient

GUS gene that has been expressed will change substrate X-Gluc into product with blue color





- Expression level of ads in the transformed *A. annua*

OD_{600}	Expression level of ads in transformed *A. annua*
0,5	1,64
0,8	2,67
1,0	3,25
C-	1

Insertion of P19 gene

A

***Post Transcriptional Gene Silencing (PTGS)* or *RNA silencing* atau RNAi (RNA interference)**

B

C

protective response of antivirus in eucariyotik

→ Inhibit virus replication in plants

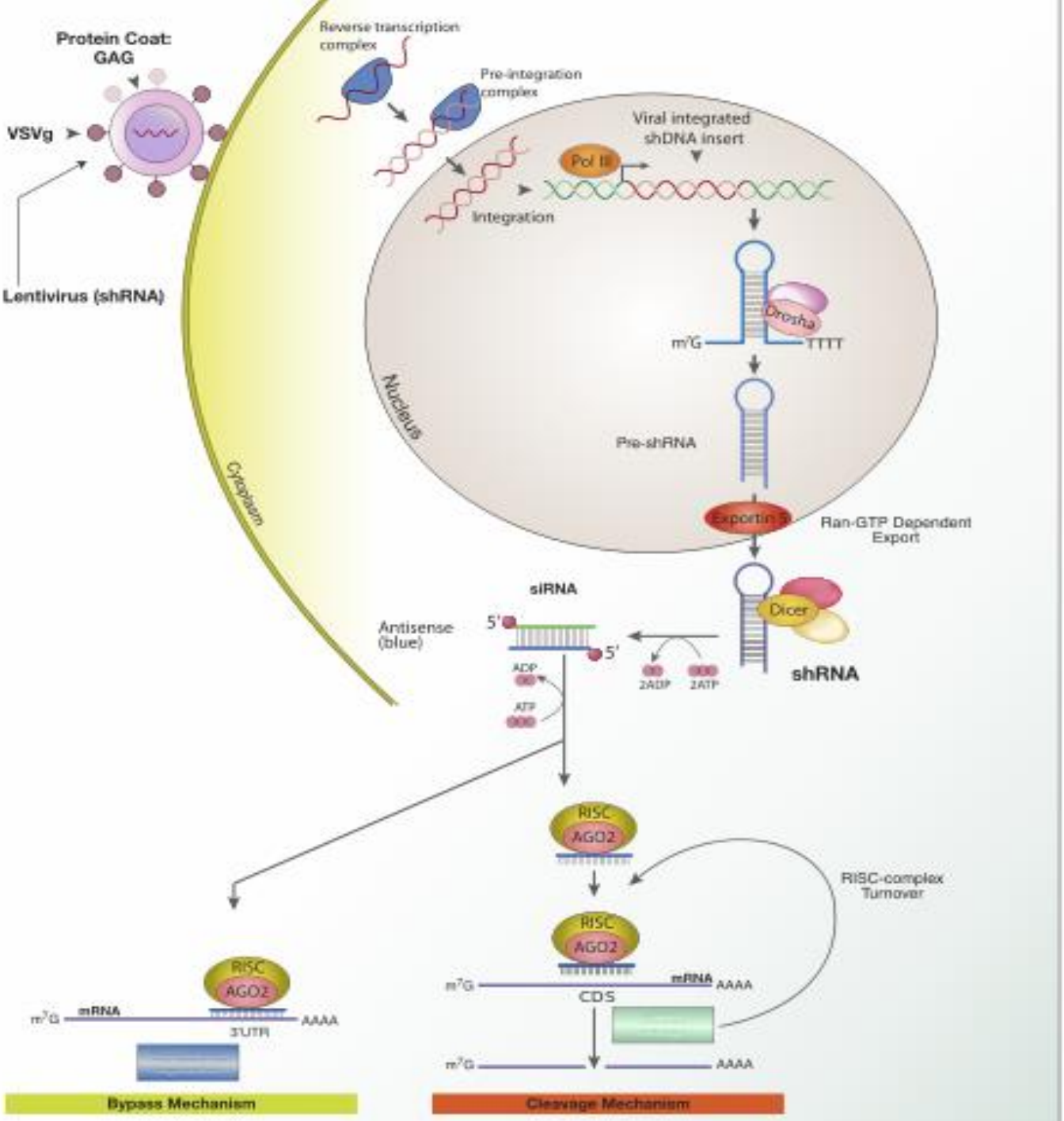
→ Inhibit eficiency of trasient expression in *Agrobacterium based tranformation*

D

E

Voinnet, 2003. Voi nnet 2001 (***Takeda et al,. 2002***)

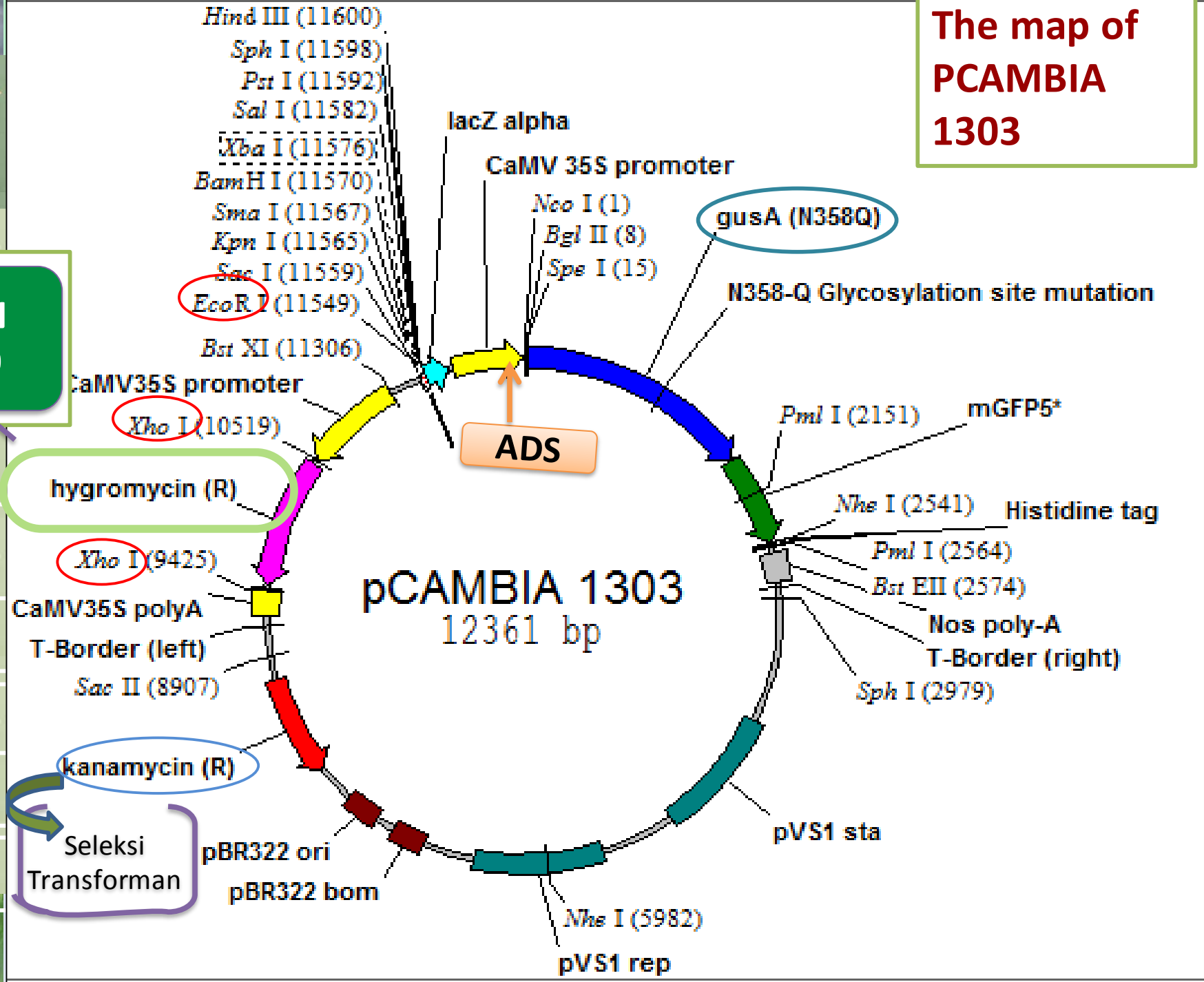
Mechanism RNAi



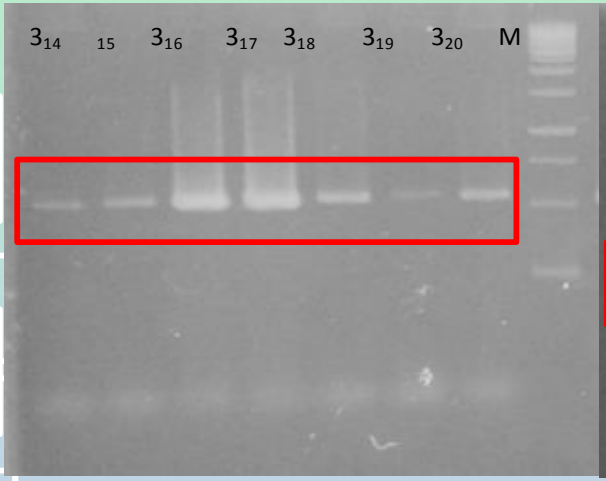
Voinnet, 2003. Voi nnet 2001 (*Takea*

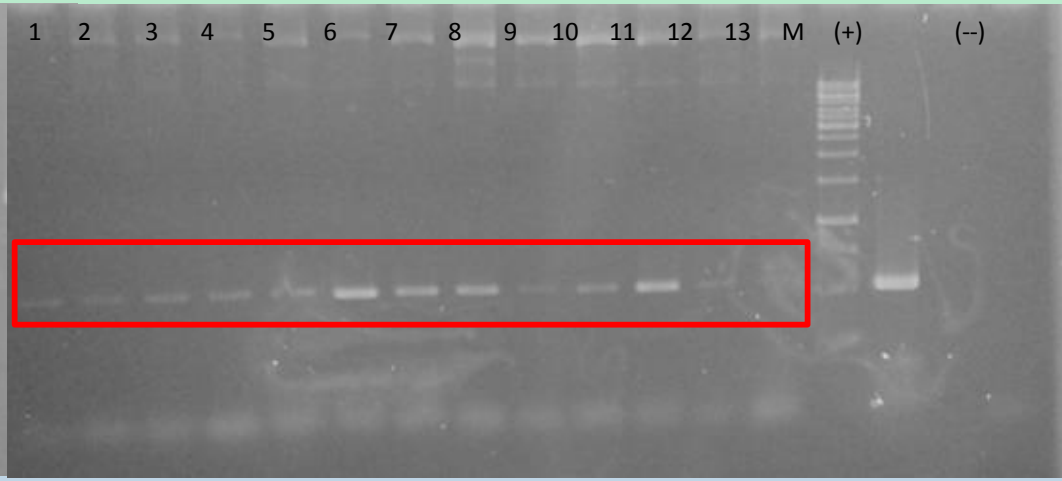
The map of PCAMBIA 1303

GEN P19

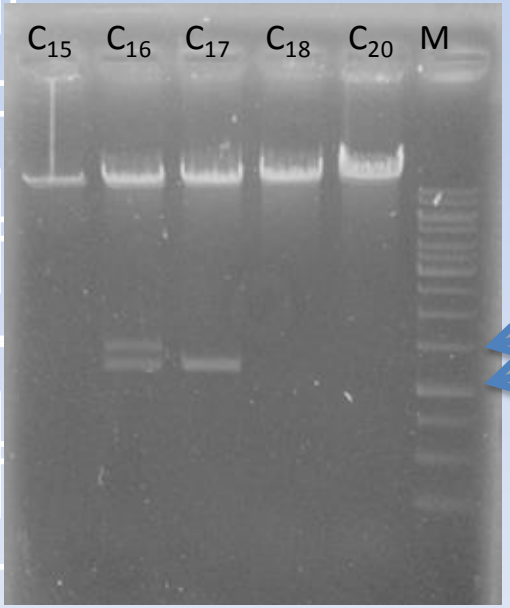


Gel electrogram of pCAMBIA_ads_p19





Restriction analysis



Sequensing

Sequence is identic with p19 gene from gene bank NCBI

A B C D E F

D_{1c}

TRANSFORMATION OF PLASMID INTO ARTEMISIA ANNUA Via *A. TUMEFACIENS*

- Transformed plasmids

1. Empty pCAMBIA 1303 –(negative control)
2. pCAMBIA 1303-ads
3. pCAMBIA 1303-p19
4. pCAMBIA 1303-ads-p19
5. pCAMBIA 1303-ads cotransfromation with pCAMBIA 1303-p19

Transformation procedure

Suspension of A. tumefaciens containing each plasmid + Acetosyringone + Siluet S 408

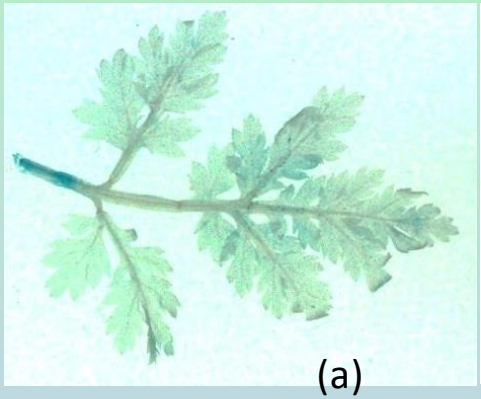
Incubate the leaves of *Artemisia annua* for 20 minutes with vacuum infiltration methods in dark

A

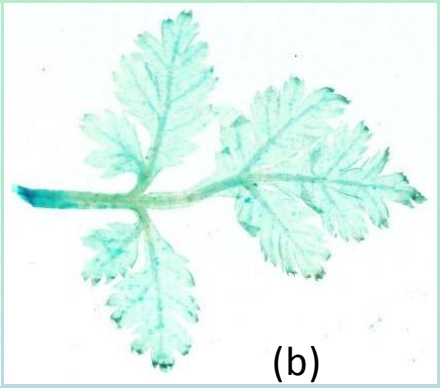
B

C

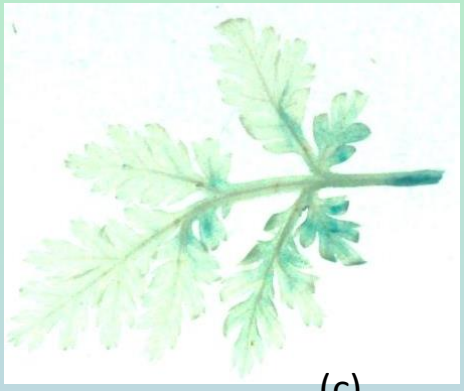
D_3



(a)

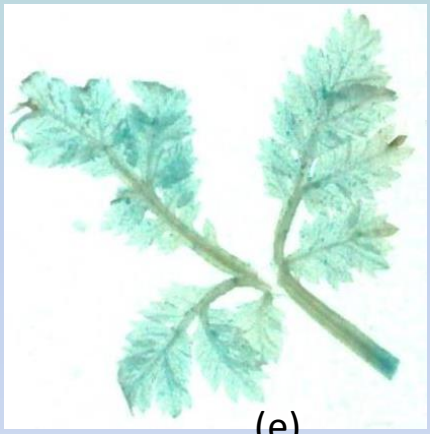


(b)



(c)

(d)



(e)

(a) plasmid pCAMBIA 1303 (*wild type)*
(b) plasmid recombinant pCAMBIA 1303-*ads*
(c) plasmid recombinant pCAMBIA 1303-*p19*
(d) plasmid recombinant pCAMBIA 1303-*ads-p19*
(e) Co-transformation plasmid recombinan pCAMBIA 1303-*ads* and pCAMBIA 1303-*p19*

No	Samples (transformed leaves)	Total Area	Blue Area	Percentage of blue area (%)	Rata-rata (%)
a	PCAMBIA 1	1547503	80990	5,23	13,70 ± 7,39
	pCAMBIA 2	1509154	285278	18,90	
	pCAMBIA 3	680527	115378	16,95	
b	pCAMBIA-ads 1	976061	879248	90,08	48,59 ± 38,90
	pCAMBIA-ads 2	842861	360786	42,80	
	pCAMBIA-ads 3	904368	116577	12,89	
c	pCAMBIA-p19 1	74398	33657	45,24	24,99 ± 17,65
	pCAMBIA-p19 2	70712	11942	16,89	
	pCAMBIA-p19 3	31112	3994	12,84	
d	pCAMBIA-ads- p19 1	47797	21140	44,23	70,89 ± 23,61
	pCAMBIA-ads-p19 2	39358	35095	89,17	
	pCAMBIA-ads-p19 3	46189	36616	79,27	
e	co-transformation1	56196	54135	96,33	72,49 ± 22,16
	co-transformation 2	475974	250039	52,53	
	co-transformation 3	63854	43807	68,60	

Tanpa *Agrobacterium rhizogenes* (1)

Dengan *Agrobacterium rhizogenes* (2)

Agrobacterium rhizogenes tanpa pCAMBIA (1)

Agrobacterium rhizogenes tanpa pCAMBIA (2)

pCAMBIA-*p19* 1

pCAMBIA-*p19* 1

pCAMBIA-*ads* 1

pCAMBIA-*ads* 2

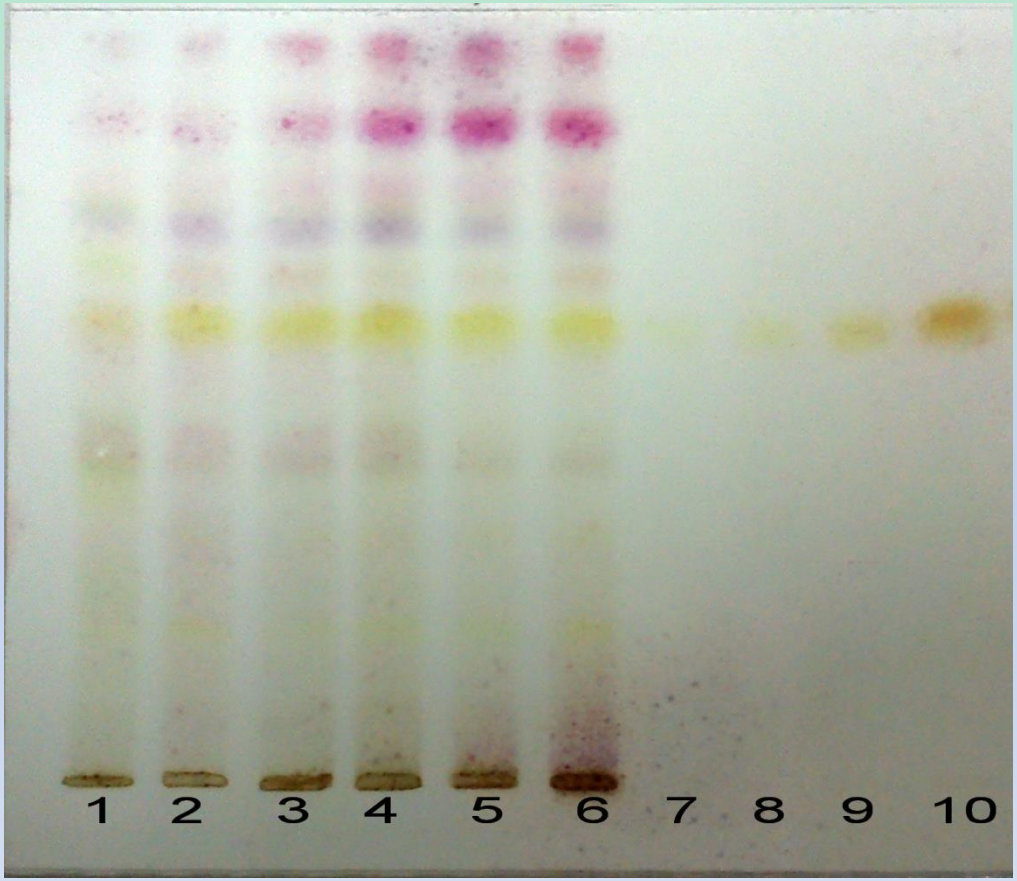
pCAMBIA-*p19-ads* 1

pCAMBIA-*p19-ads* 2

Ko-transformasi 1

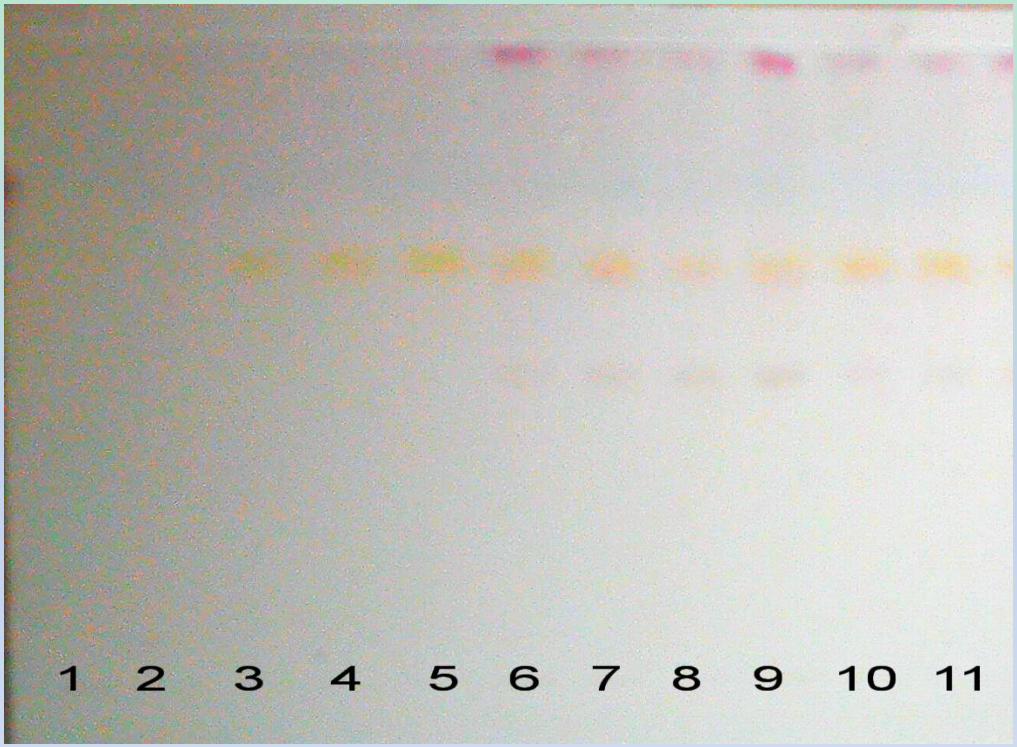
Ko-transformasi 2

Analysis of artemisinin from transformed A. annua Leaves



Note: 1) AGL only 2) empty pCAMBIA (negative control), 3) pCAMBIA_ads, 4) PCAMBIA_p19, 5) pCAMBIA_ads+p19, 6) cotransformation pCAMBIA ads and pCAMBIA_p19, 7-10) artemisinin standard 30, 50, 100 and 300 ppm respectively

Analysis of artemisinin from transformed A. annua hairy root



Note: 1-5) artemisinin standard 10, 20, 30, 40 and 50 ppm respectively, 6) AGL only 7) empty pCAMBIA (negative control), 8) pCAMBIA_ads, 9) PCAMBIA_p19, 10) pCAMBIA_ads+p19, 11) cotransformation pCAMBIA ads and pCAMBIA_p19,

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