Our four most important natural resource is the air, water, oil, and rubber.

Creating the domestic crop, producing NATURAL RUBBER FOR INDUSTRY, by the CRISPR/Cas Nuclease RNA-guided sunflower genome editing and the sunflower chloroplast genetic transformation

Alexander K. Gaponenko

Professor of Genetics,

Chief Researcher of Koltzov Institute of developmental biology RAS

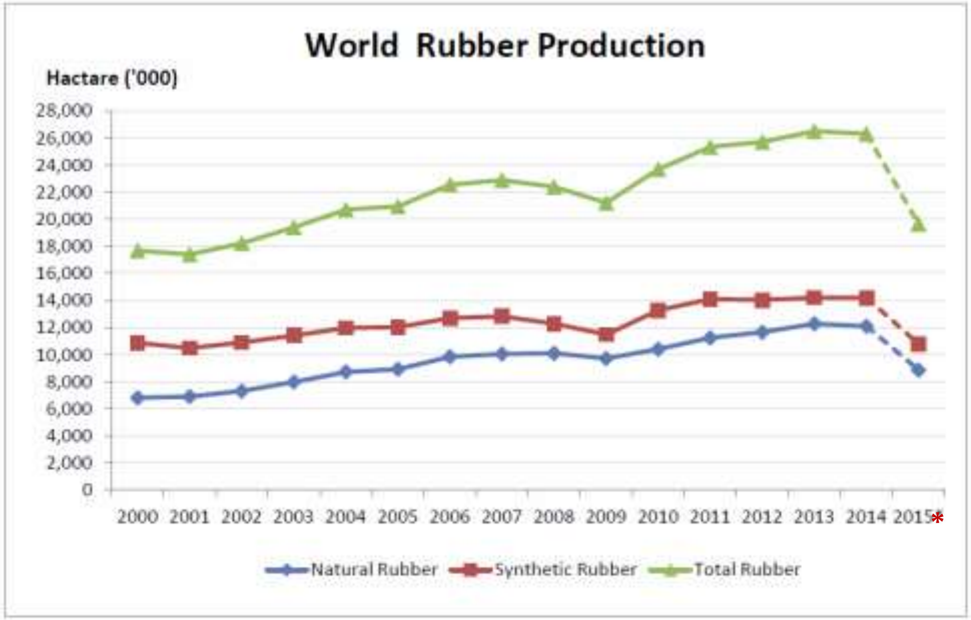
The world market of natural rubber

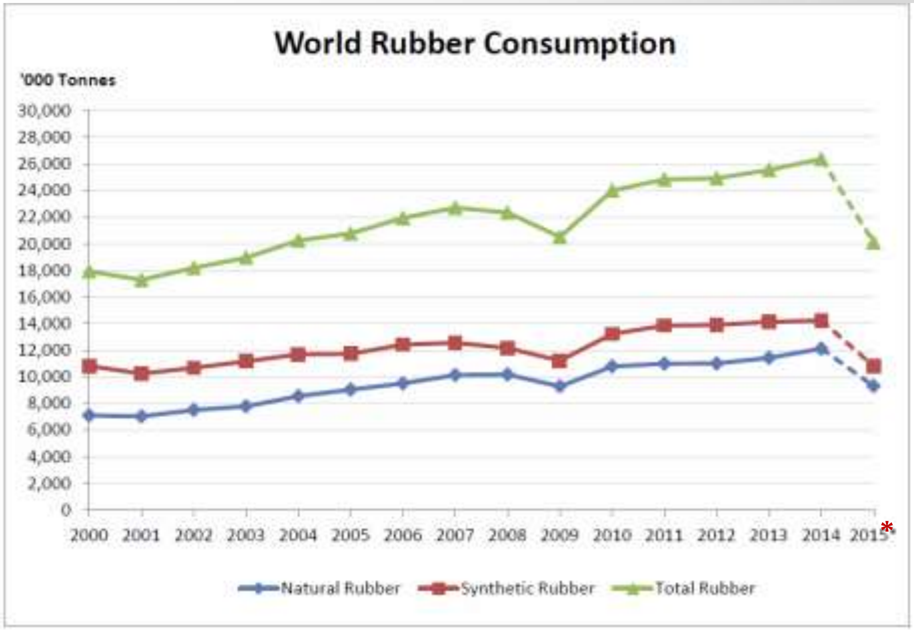
Produced in 2014 total 12.070 million tones = $ 25,23 billion.
Consumed in 2014 total 12.159 million tones = $ 25,41 billion

These and some other rubber products 40000 is derived from a tropical Brazilian rubber tree *Hevea brasiliensis.* But other kinds of plants in 2500, including such as goldenrod, guayule, dandelion and sunflower also produce rubber. Most plants do not make it easy enough and cheap to cover the cost of collection and processing.

Production	From the world,%	Consumption	From the world,%
Thailand	33%	USA	17.5%
Indonesia	26%	Europe	16.3%
Malaysia	20%	China	13%
Vietnam		Japan	11%
INDIA		Korea	5%

Natural Resources Natural rubber is not sufficient to fully meet the rapidly growing demand in it.





* *January-September*

Year	Rubber Production ('000 tonnes)		
	Natural Rubber	Synthetic Rubber	Total Rubber
2000	6,811	10,870	17,681
2001	6,913	10,483	17,396
2002	7,317	10,906	18,223
2003	7,986	11,414	19,400
2004	8,726	11,979	20,705
2005	8,921	12,025	20,946
2006	9,850	12,700	22,550
2007	10,057	12,829	22,886
2008	10,098	12,285	22,383
2009	9,723	11,488	21,210
2010	10,403	13,277	23,680
2011	11,239	14,091	25,330
2012	11,658	14,042	25,700
2013	12,281	14,214	26,495
2014	12,103	14,205	26,308
2015*	8,862	10,790	19,652

Year	Rubber Consumption ('000 tonnes)		
	Natural Rubber	Synthetic Rubber	Total Rubber
2000	7,108	10,830	17,938
2001	7,039	10,253	17,292
2002	7,515	10,679	18,194
2003	7,797	11,177	18,973
2004	8,562	11,693	20,255
2005	9,049	11,731	20,780
2006	9,513	12,434	21,947
2007	10,138	12,576	22,714
2008	10,187	12,173	22,360
2009	9,289	11,228	20,517
2010	10,792	13,225	24,017
2011	10,997	13,856	24,853
2012	11,013	13,915	24,927
2013	11,430	14,136	25,566
2014	12,125	14,252	26,377
2015*	9,314	10,797	20,111

***Source:* International Rubber Study Group (IRSG)**

Over the years, researchers have studied the potential of plant species that are suitable for the production of natural rubber:

Agronomic and natural rubber characteristics of sunflower as a rubber-producing plant

Calvin H. Pearson [a], Katrina Cornish [b], Colleen M. McMahan [b], Donna J. Ratha, Jenny L. Brichta [b], Jennifer E. Van Fleet [b]

Industrial Crops and Products 31 (2010) 481–491

[a] *Colorado State University, Agricultural Experiment Station, Western Colorado Research Center, 1910 L Road, Fruita, CO 81521, United States* [b] *USDA-ARS, Western Regional Research Center, 800 Buchanan St., Albany, CA 94710, United States*

The Rationale for Transforming Sunflower into a Rubber-Producing Crop

C.H. Pearson*, K. Cornish et al, Issues in new crops and new uses. 2007. J. Janick and A. Whipkey (eds.). ASHS Press, Alexandria, VA.»

The potential for sunflower as a rubber producing crop for the united states

Cornish, K., Pearson,C.H., Rath,D.J., Dong, N., McMahan, C.M., Whalen, M. HELIA, (2007) 30, Nr. 46, p.p. 157-166,

20 YEARS AGO LAUNCHED THE RACE FOR THE CREATION OF ALTERNATIVE HEVEA PLANT SOURCE OF NATURAL RUBBER, WHICH INVOLVES UNIVERSITIES AND COMPANIES OF DEVELOPED

	Universities, Research Centers	Companies	Countries	Year Год
1.	United States Department of Agriculture, ARS Western Regional Research Center in Albany, California.	Yulex Corporation	USA	March 1995
2.	Colorado, Ohio, Oregon, Guelph, Nevada, Washington, Akron	Cooper Tire & Rubber Company; Bridgestone; Ford	USA, Japan	1993
3.	Korea Research Institute of Bioscience & Biotechnology; Korea University, Seoul	Kumho Life and Environmental Science Laboratory	South Korea	2002
4.		Kultevat Inc., KeyGene; Sumitomo Rubber Industries	USA, Netherlands, Japan	2013
5.		KeyGene и Kok-Saghyz TM	Netherlands, Kazakhstan	2014
6.	Fraunhofer Institute for Molecular Biology and Applied Ecology IME	MITAS; Continental Tires	Germany	2010
7.	EU-based Production of Alternative Rubber and Latex Sources: Wageningen UR, AFSG – Biobased Products; Czech Academy of Sciences, Institute of Botany; Basque Institute for Agrarian Research and Development; Centre de coopération internationale en recherché agronomique pour le développement	Bayer Crop Science, Bayer Bio Science;	Belgium, Netherlands, France, Czech Republic, Spain, France	2006

Comparison of methods of farming, amount and properties of rubber from producers, the choice of object

№	RUBBER PLANT	Period before collection of the NR	AGROTECHNICS FOR CULTIVATION AND HARVESTING	RUBBER OUTPUT KG / HA / YR	Properties molecular weight NC (kDa)
1.	**Hevea brasiliensis** *(Hevea brasiliensis* (Willd. ex A.Juss.) Müll.Arg.*)*	8 years	Hand-consuming With each notch is obtained about 30 mL of latex	**500 – 3000** Dry raw rubber	1,310
2.	**Russian dandelion** *(Taraxacum kok-saghyz* Rodin) *(Taraxacum brevicorniculatum* Korol*)*	1 - 2 years	Farming used manual labor to collect seeds, harvesting mechanized	**100 - 500** On average, 15% of the weight of the root. Sometimes up to 24% NR. The harvest Roots to 1.5 t / ha.	2,180
3.	**Tau saghyz** *(Scorzonera tausaghyz)*	3 years	Farming used manual labor to collect seeds, harvesting mechanized	**150 - 250** In the roots of 1 year is 6-7% NR, 2 years of 5-10%, 12-18% of 3-4 year olds, 35-40% of long-term. Plants of 4 years root mass is 34-70 g	No data
4.	**Crimea saghyz** *(Taraxacum hybernum)*	1 - 2 years	Farming used manual labor to collect seeds, harvesting mechanized	Нет данных	No data
5.	**Guayule** *(Parthenium argentatum,)*	4 years	Farming is technologically effective	**8 - 10%** Rubber on the dry weight yield	1,280
6.	**Sunflower** *(Helianthus annuus* L*)*	1 year	Farming is highly technological	**To 1.6%** on the yield of dried leaves. At high (3-4 m) varieties yield of green mass of up to **100 t / ha**	The two fractions of 600 and 66

“Production of rubber in transgenic sunflower possibly to establish only through genetic engineering methods “

Source: (ALTERNATIVE SOURCES OF NATURAL RUBBER, Outputs from the EPOBIO project, November 2006, Prepared by Dr. Jan van Beilen)

A COLLABORATIVE RESEARCH PROJECT OF THE USDA AND SEVERAL UNIVERSITIES WAS INITIATED IN 2001 IN THE USA
TO TRANSFORM SUNFLOWER INTO A RUBBER-PRODUCING CROP.

The overall objectives of this research project on sunflower were to:

1. **Insert genes to optimize rubber synthesis into sunflower;**
2. **Develop the agronomy for genetically-engineered, rubber-producing sunflower;**
3. **Evaluate genetically-engineered, rubber-producing sunflower for insect and disease pests when grown under cultivated conditions;**
4. **Develop crop enterprise budgets, and processing and product analysis for genetically modified,rubber-producing sunflower;**
5. **Identify and partner with agribusinesses, farmer organizations, and private industries for crop production, processing, and utilization of rubber and co-products produced from**

Source: «The Rationale for Transforming Sunflower into a Rubber-Producing Crop», C.H. Pearson, K. Cornish et al, in "Issues in new crops and new uses", 2007, J. Janick and A. Whipkey (eds.). ASHS Press, Alexandria».

SUNFLOWER RESEARCH PROJECTS STARTED IN 2001 IN THE UNITED STATES SEEMS TO HAVE FAILED WHEREAS:

1. Attempts to stable nuclear genetic transformation of sunflower in a number of laboratories have been unsuccessful. It was one publication, rather uninformative.
2. Method of chloroplast transformation of sunflower has not been developed.
3. Therefore biotech companies in the USA (Kultevat Inc.), in the Netherlands (KeyGene) and Germany (MITAS; Continental Tyres) are actively working with the cook-sagyz.
4. In the city of Wooster, Ohio, United States operates a pilot plant for the production of rubber cook sagyz.
5. Research Center, Ohio State University (OARDC) received a grant of $ 3 million, together with industrial partners to develop a process for producing rubber from the cook-sagyz.

Fig. 1. & Fig. 2. *Taraxacum kok-saghyz,*
Fig. 3. Professor KlEinheinz, head work in OARDC, USA.
Fig. 4. Professor Dirk Prüfer, Institute for Biology and Biotechnology of Plants, Münster, Germany
Fig. 5. Latex in the fault root Kok sagyz
Fig. 6. Crops of Kok sagyz in Wooster, Ohio, USA.

Sources: Issues in new crops and new uses. 2007. J. Janick and A. Whipkey(eds.). ASHS Press, Alexandria, VA. http://cornishlab.cfaes.ohio-state.edu

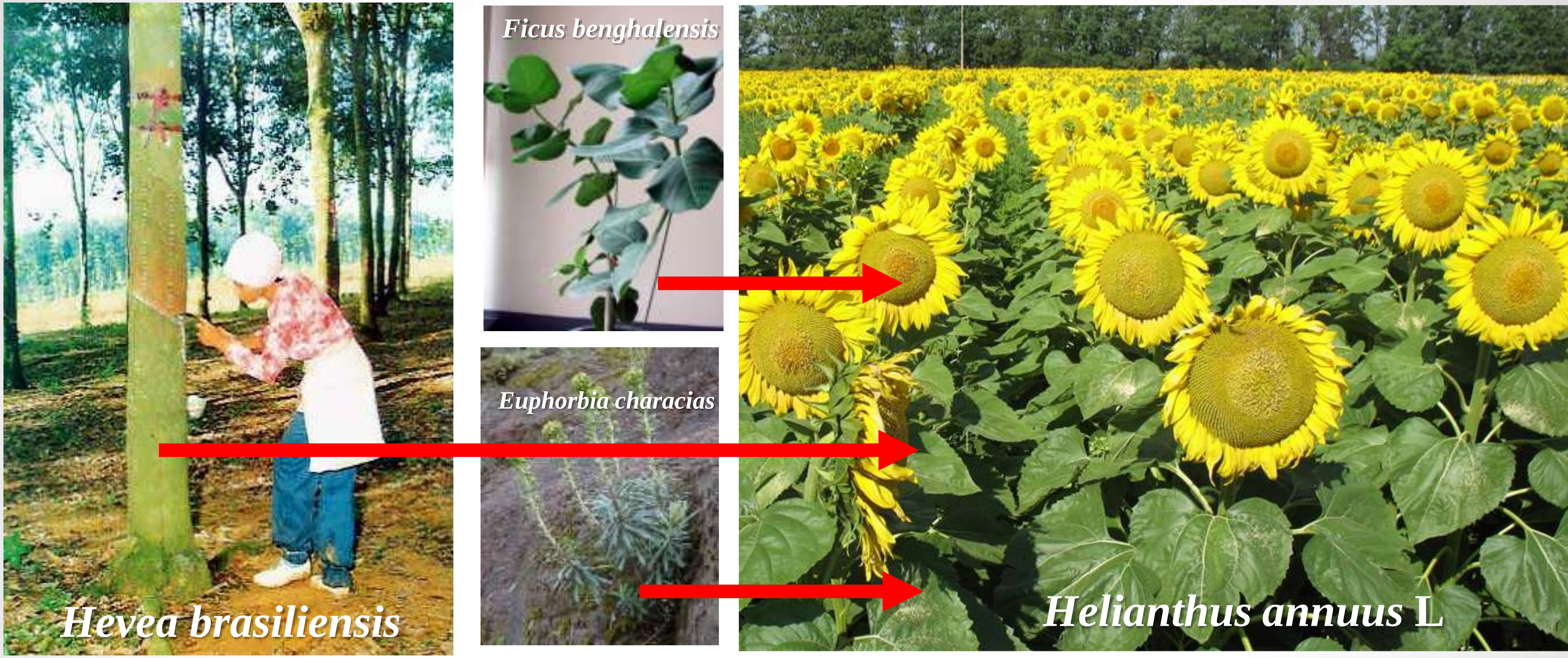
Ideally suitable for the production of natural rubber (NR) crops should:

1. Grow rapidly in the climatic conditions of the Russia
2. Have a large biomass
3. Maintain a high % of dry matter in parts of the plant that contains NR
4. Be for alternating annual crops with other crops for satisfaction of the needs of agricultural activities of region
5. Fit the mechanized plant cultivation.
6. Have proven agricultural technologies of cultivation

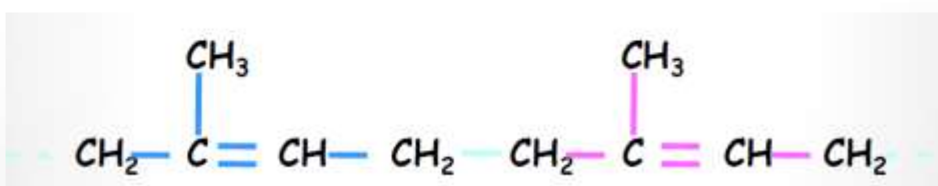
1 - Sunflower
2 – Guayule
3 – Russian dandelion
4 - Tau saghyz
5 - Jerusalem artichoke

Source: The Rationale for Transforming Sunflower into a Rubber-Producing Crop . C.H. Pearson*, K. Cornish et al, Issues in new crops and new uses. 2007. J. Janick and A. Whipkey (eds.). ASHS Press, Alexandria, VA.

GENETICALLY ENGINEERED TRANSFER GENES OF RUBBER PLANTS INTO SUNFLOWER AND INCREASE EXPRESSION OF THEIR OWN GENES SYNTHESIS OF RUBBER IN THE CHLOROPLAST



Natural rubber (cis-1,4-polyisoprene). It is synthesized 2,000 kinds of plants (sunflower rubber content of about 1%). Natural rubber is a high molecular weight hydrocarbon isoprene polymer whose composition corresponds to the formula (C5H8)n, cis-isoprene resin:



Prof. Alex K. Gaponenko, Koltzov institute of development biology RAS, Moscow

IT IS CLEAR THAT DESPITE THE GREAT ACHIEVEMENTS OF GENETICS, MOLECULAR BIOLOGY AND SYNTHESIS CONTROL OF THE NR IN PLANTS, A FEW ATTEMPTS TO STABLE NUCLEAR GENETIC TRANSFORMATION OF SUNFLOWER IN THE USA HAVE NOT VERY SUCCESSFUL YET & METHOD FOR THE CHLOROPLAST GENOME OF SUNFLOWER TRANSFORMATION IS NOT DEVELOPED YET …

THEREFORE, SCIENTISTS AND BIOTECH COMPANIES OF THE USA GERMANY AND THE NETHERLANDS DREW ATTENTION TO THE KOK-SAGHYZ (RUSSIAN DANDELION)

April 8, 2013
View More: http://arviddewindt.pass.us/keygene-portretten

Today the US based biotech company Kultevat and KeyGene, a biotech company from the Netherlands announced that they entered into collaboration for production of rubber based on the Russian dandelion. This plant has demonstrated potential as a domesticated crop for the U.S. and Europe; the origin of the species is the south eastern part of…

Press release: KeyGene and Kok-Saghyz TM signed agreement Kazakh dandelion

On March 23, 2014 KeyGene and Kok-Saghyz TM (KSTM), a Kazakhstan-based company, signed an agreement that forms the start of a long-term strategic partnership on the production of natural rubber from the Kazakh dandelion. The agreement was signed in presence of Mr. Hans Driesser, Ambassador of the Kingdom of The Netherlands to Kazakhstan, Kyrgyzstan, and Tajikistan and Mr. Asset Issekeshev, Deputy Prime-Minister, Minister of Industry and New Technologies of the Republic of Kazakhstan.

Comparison of the transgenic sunflower field test releases with another transgenic crops field test releases around the world (1996 - 2016).

Despite the fact, that one of the first transformations of the plant cell was made on sunflower (Timothy Hall et al, 1983; Matzke M.A., et al 1984), & high economic value of sunflower, genetic engineering of sunflower remains behind the all main crops.

Country / Crop	USA 2015	Canada 2014	EU 2012	Total 2015
Maize	8746	371	936	10053
Rapeseed	351	1996	381	2738
Potato	1040	330	307	1677
Soybean	2553	100	19	2672
Cotton	1219	-	91	1310
Wheat	559	382	36	977
Tomato	773	-	75	848
Wheat	240	29	282	551
Rice	343	-	36	379
Barley	113	12	13	138
Sunflower	32	2	15	49

Countries		Number of field trials
1.	USA	32
2.	France	10
3.	Canada	2
4.	Netherlands	2
5.	Spain	3
6.	Argentine	?

http://www.gmo-compass.org/eng/database/plants/68.sunflower.html

http://www.nbiap.vt.edu/search-release-data.aspx
http://gmoinfo.jrc.ec.europa.eu/overview/dbplants.asp
http://www.inspection.gc.ca/plants/plants-with-novel-traits/approved-under-review/field-trials/eng/1313872595333/1313873672306#sum

Our RF patents on methods of the genetic transformation of sunflower, issued in 2000 and 2001 are still maintained in force

РОССИЙСКАЯ ФЕДЕРАЦИЯ

ПАТЕНТ

НА ИЗОБРЕТЕНИЕ

№ 2179187

Российским агентством по патентам и товарным знакам на основании Патентного закона Российской Федерации, введенного в действие 14 октября 1992 года, выдан настоящий патент на изобретение

СПОСОБ ПОЛУЧЕНИЯ ТРАНСГЕННЫХ РАСТЕНИЙ ПОДСОЛНЕЧНИКА

Патентообладатель(ли):

Гапоненко Александр Константинович

по заявке № 2000132894, дата поступления: 28.12.2000

Приоритет от 28.12.2000

Автор(ы) изобретения:

Гапоненко Александр Константинович

Патент действует на всей территории Российской Федерации в течение 20 лет с **28 декабря 2000 г.** при условии своевременной уплаты пошлины за поддержание патента в силе

Зарегистрирован в Государственном реестре изобретений Российской Федерации

г. Москва, ***10 февраля 2002 г.***

Генеральный директор

А.Д. Корчагин

РОССИЙСКАЯ ФЕДЕРАЦИЯ

ПАТЕНТ

НА ИЗОБРЕТЕНИЕ

№ 2193066

Российским агентством по патентам и товарным знакам на основании Патентного закона Российской Федерации, введенного в действие 14 октября 1992 года, выдан настоящий патент на изобретение

БАЛЛИСТИЧЕСКИЙ СПОСОБ ПОЛУЧЕНИЯ ТРАНСГЕННЫХ РАСТЕНИЙ ПОДСОЛНЕЧНИКА (Helianthus annuus L.)

Патентообладатель(ли):

Гапоненко Александр Константинович

по заявке № 2001129129, дата поступления: 30.10.2001

Приоритет от 30.10.2001

Автор(ы) изобретения:

Гапоненко Александр Константинович

Патент действует на всей территории Российской Федерации в течение 20 лет с **30 октября 2001 г.** при условии своевременной уплаты пошлины за поддержание патента в силе

Зарегистрирован в Государственном реестре изобретений Российской Федерации

г. Москва, ***20 ноября 2002 г.***

Генеральный директор

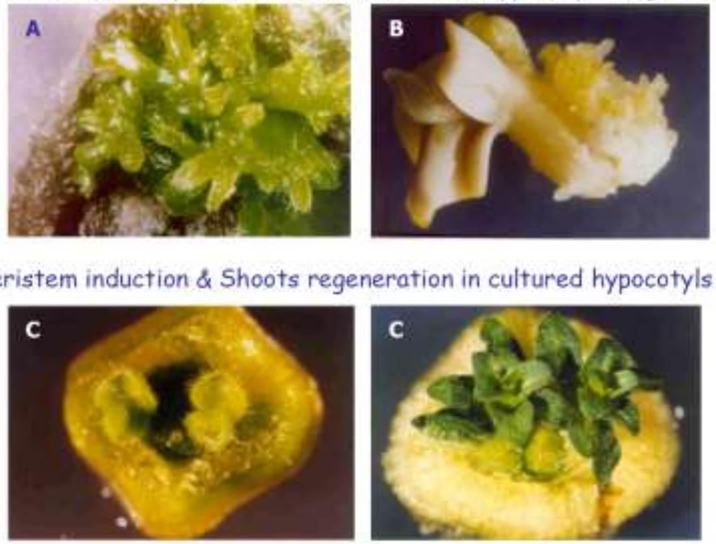
А.Д. Корчагин

Prof. Alex K. Gaponenko, Koltzov institute of development biology RAS, Moscow

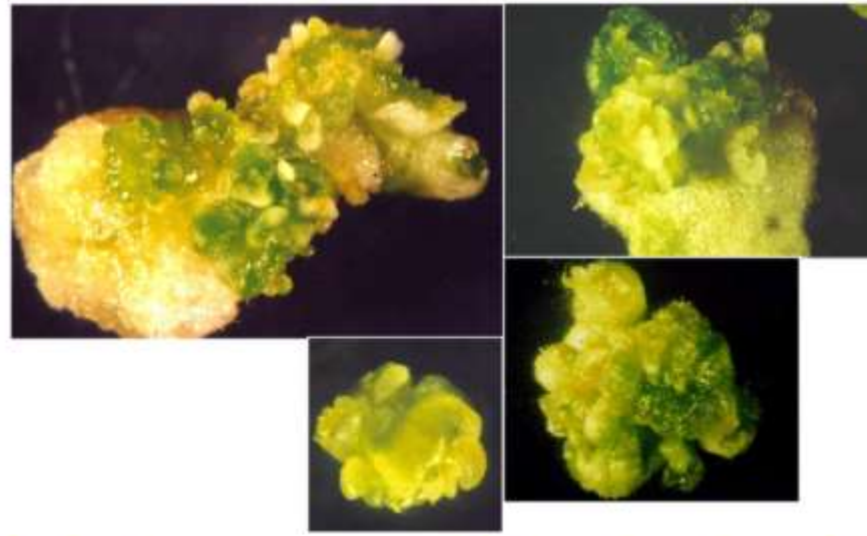
How we are doing sunflower transformation

Regeneration of shoots & embryoids from A – cotyledons, B – hypocotyl (or immature embryo) & C - cultured *in vitro* hypocotyl's segments

Apical meristem induction & Shoots regeneration in cultured hypocotyls segmen

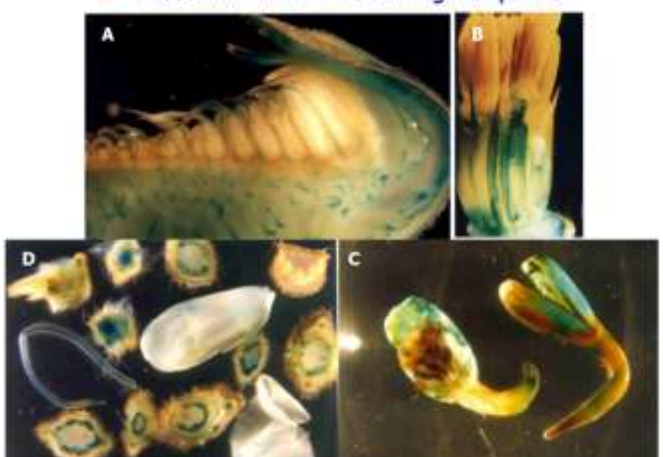


Multiple sunflower shoots (apical meristems) induction *in vitro*.



Marker *uidA (GUS)* gene expression in the transgenic sunflower:

A - Inflorescence cut; B - flowers with developing seeds; C - germinating seeds of the T_1 (2nd generation) & D - different tissues of transgenic plant.



Affect of genotypes and different vectors on the frequency of Sunflower genetic transformation

Agrobacterium Transformation Frequency, %

Genotype	Binary Vector used	Genes introduced	Number of shoots used	Number of transformed plants obtained	Frequency of transformation, %
Peredovic	p035GUS/Intron (hgh)	*uidA* , *Hgh*	202	7	3,46
Saratovski 82	pCAMBIA1300-*cpt-1*	*cpt-1*, *Hgh*	165	6	3,60
Skorospely 87	pCAMBIA1300-*cpt-1*	*cpt-1*, *Hgh*	233	1	0,42
Skorospely 87	pCAMBIA1300-*HMGR*	*HMGR*, *Hgh*	420	2	0,46
Saratovski 87	pHBT59-*HAM 59*	*HAM 59*, *Hgh*, *NptII*	2080	4	0,10

Biolistic Transformation Frequency, %

Genotype	Vector used	Gene introduced	Number of shoots used	Number of Transformed plants obtained	Frequency of transformation
Peredovik	pRTL2-hgh	*hgh*	300	8	2,66

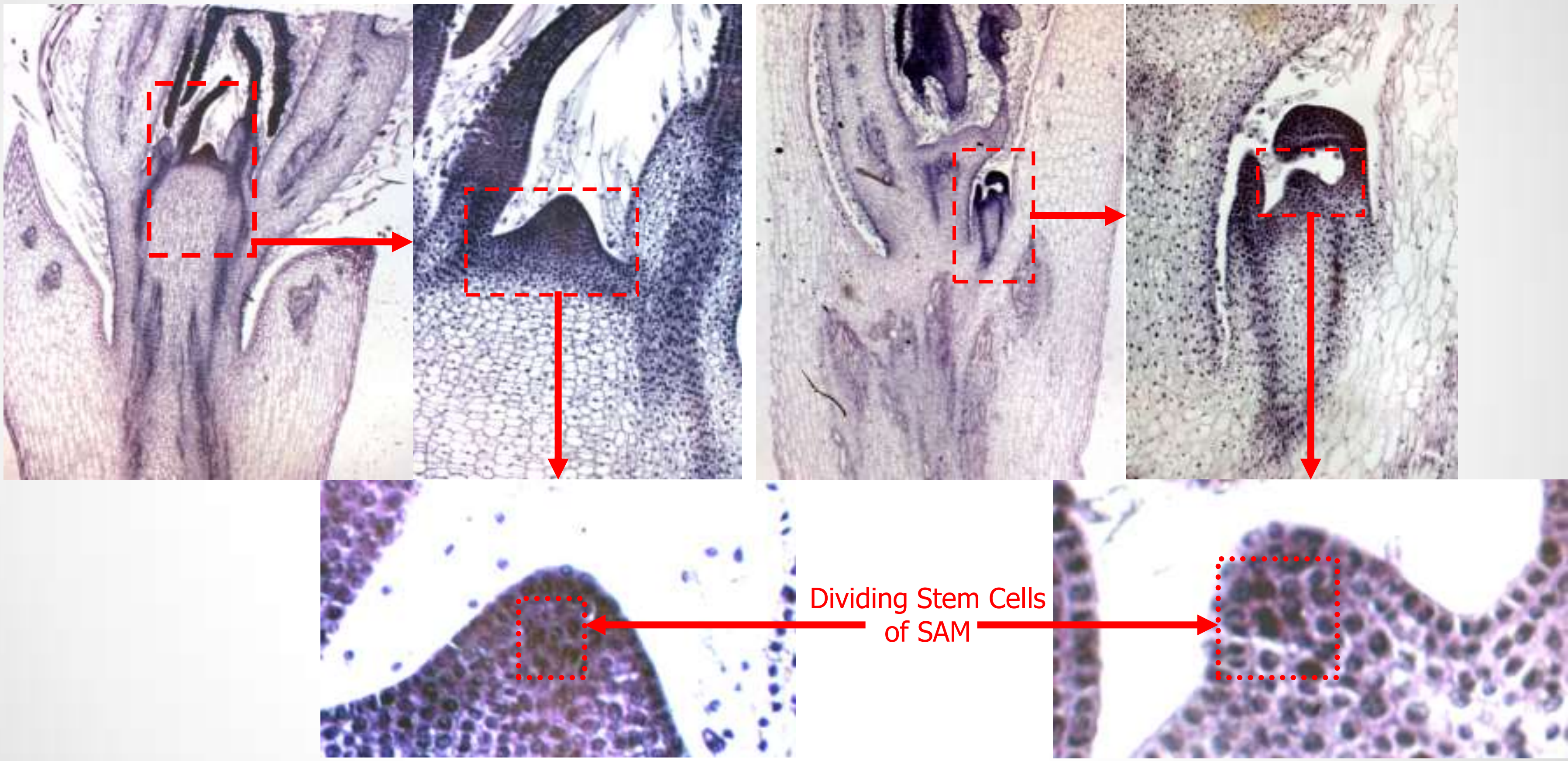
Cpt-1 - Cis-prenyl transferase from *Hevea brasiliensis*
HMGR - 3-Hydroxi3-methylglutaryl-CoA reductase from *Arabidopsis thaliana*
} Genes of natural rubber biosynthesis
HAM 59 - Helianthus Annuus MADS gene of sunflower.

Prof. Alex K. Gaponenko, Koltzov institute of development biology RAS, Moscow

The primary plant meristem - the shoot apical meristem (SAM)

The shoot apical meristem (apex) of germinated sunflower.

Apical meristem induction in the cultured *in vitro* hypocotyl segments.



Sunflower plants regenerated *in vitro*

Using of 7 days old seedling and 1-2 week old shoots, training person could achieve 50-70% of shoots survival.

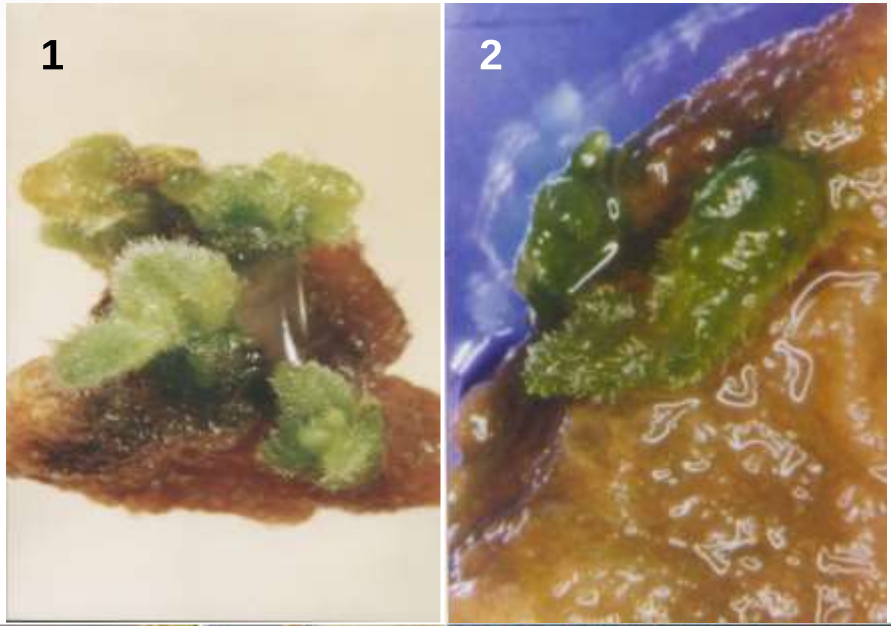
Prof. Alex K. Gaponenko, Koltzov institute of development biology RAS, Moscow

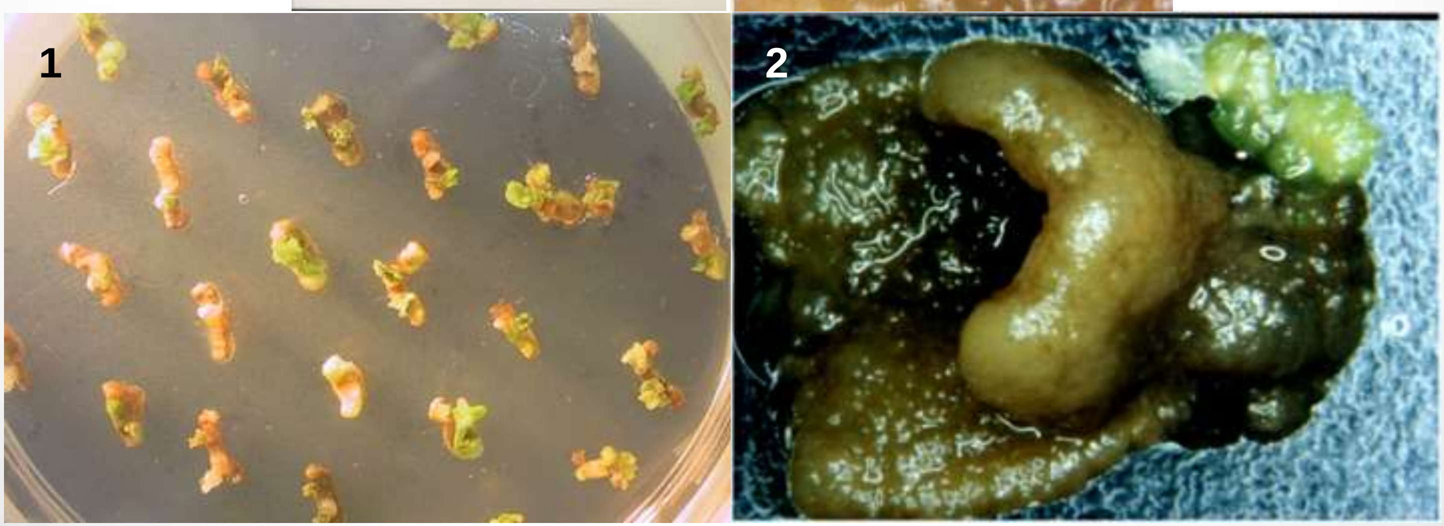
Sunflower Genetic transformation *via Agrobacteria*

A. tumefaciens line used	EHA -105 (Elizabeth Hood et al.,1993)
Binary Vectors used	p35SGUSINT; pHBT14; pHBT59; Cambia 1300; Cambia 1301;
Genes introduced	*uidA; Hyg^R; NPTII; fbp14 ; Cpt-1; HMGR; HAM 59* .
Explants	Split of 2 days old induced apical meristems, obtained in cultured hypocotyls segments. Multiples apical meristems, which have been induced *in vitro.*
Additional treatment	Explants were bombarded twice by tungsten particles using PIG.
Co-cultivation time	2-4 days on media, containing acetosyringone
Selection	On media, containing Hygromycin B, & the cefotaxime to inhibit *Agrobacterium* growth.
PCR & Southern analysis of transformed tissues	
Micropropagation of shoots passed through selection	Could be continued more then 1 month (it is allow to multiple transgenic shoots and avoid loss during grafting or rooting).
Grafting or rooting	2-3 weeks in the tube, than in the climate control chamber.
Plant recovering	Average up to 4 month for full seeds maturation.

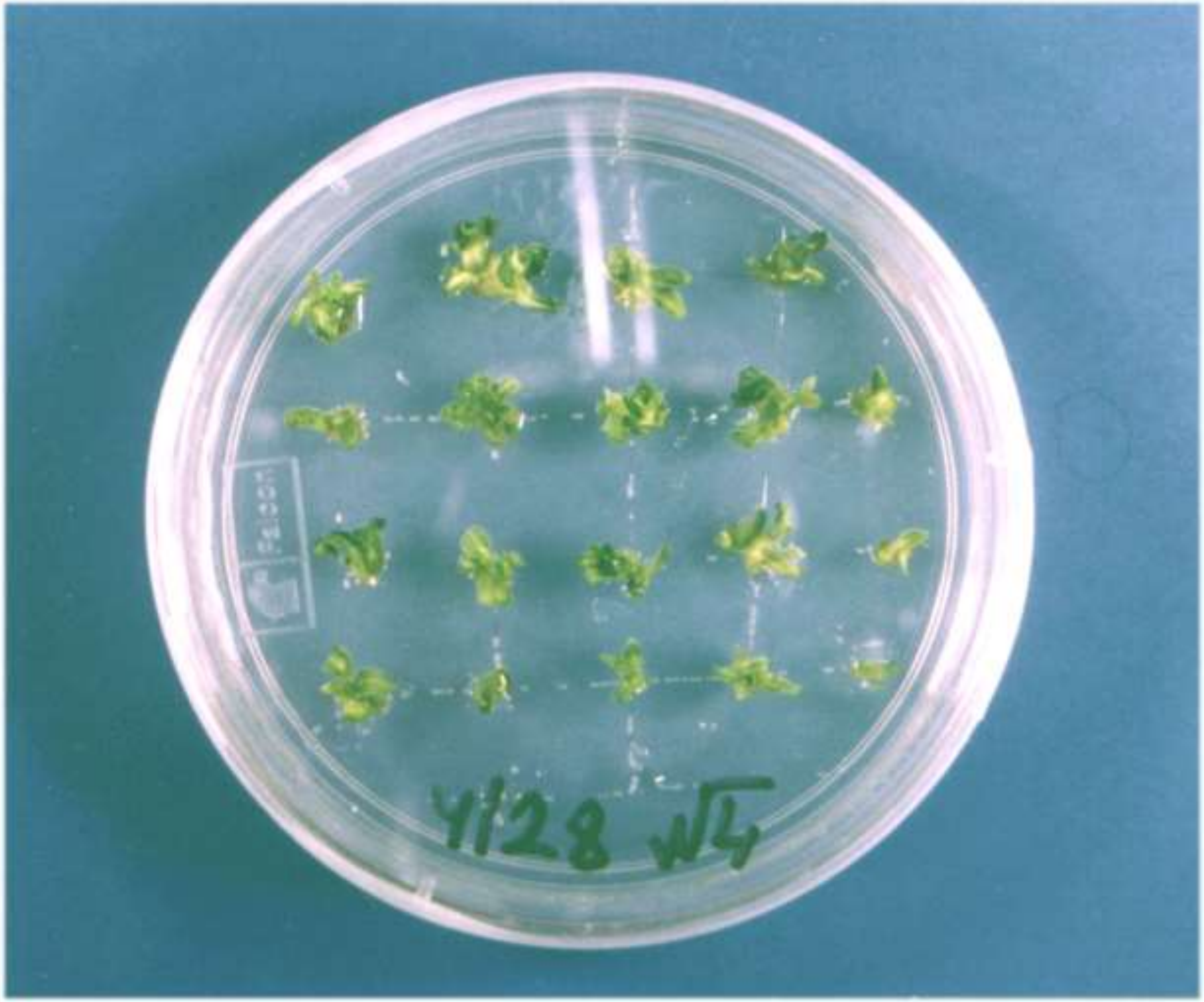
Transgenic tissue selection *in vitro*

Solid (1) or
Liquid (2) media,
containing Hygromycin B,
Time: 3 – 4 weeks.





Transgenic shoots micro propagation.



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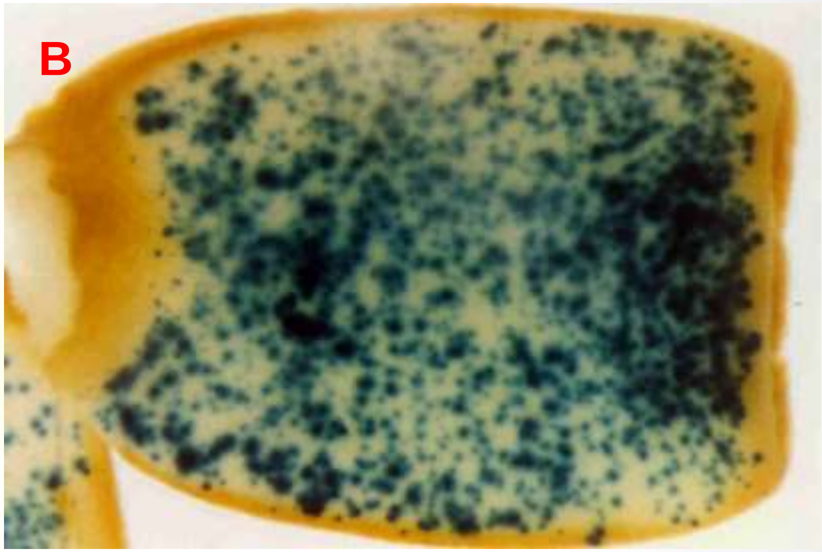
Biolistic Genetic Transformation of Sunflower

Explants	Immature embryos 1-2 mm size (5-7 days after beginning of pollination) or multiple shoots induced.
Vectors used	pRTL2- Hyg^{R} ; pRTL2-GUS; pHBT14;
Genes introduced	*Hyg^{R};, NPTII; uidA (GUS); fbp14;*
Additional treatment 1	Explants cultured on the media with high level of cytokinin
Additional treatment 2	Explants pre- & post- cultured on medium with high osmotic pressure
Additional treatment 3	Explants were bombarded twice by tungsten particles with precipitated DNA
Selection	LBM Liquid media, containing Hygromycin B
Excision of green tissue, surviving after selection	Culturing on Shoot Induction media and shoot elongation media
Micropropagation of shoots, passed through selection	Could be continued more then 1 month (it is allow to multiple transgenic shoots and avoid loss of transgenic shoots during grafting or rooting)
PCR & Southern analysis of transformed tissues	
Grafting or rooting	2-3 weeks
Plant recovering	Average up to 4 month for full seeds maturation

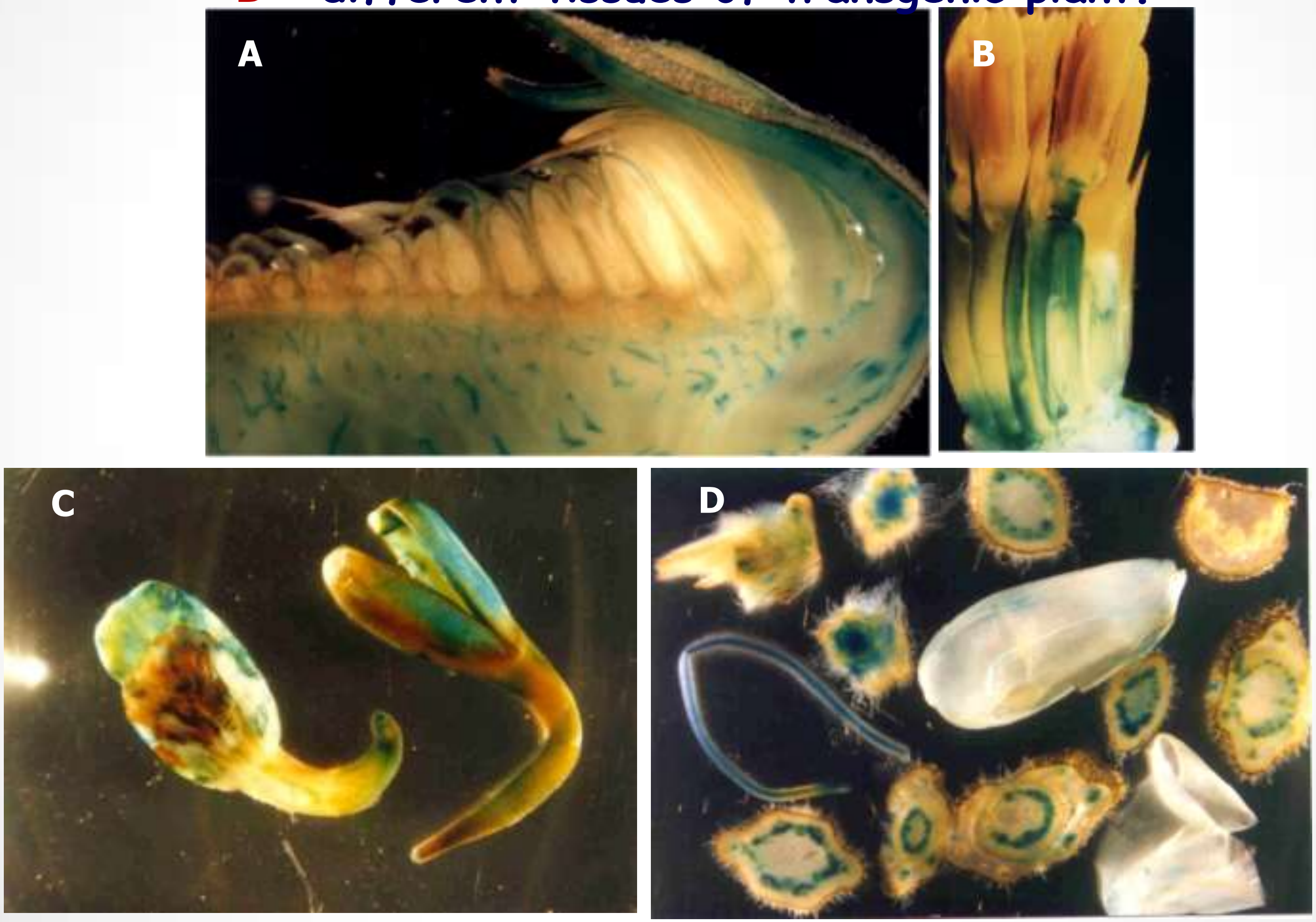
Transient expression of the marker gene - *uidA* in the A - immature embryos & B - cotyledon.



We have archived high density of gene delivery on the explants surface: 200 points per 1 immature embryo. That is mean, that at list one from 50 cells has got the expression vector with gene of interest.

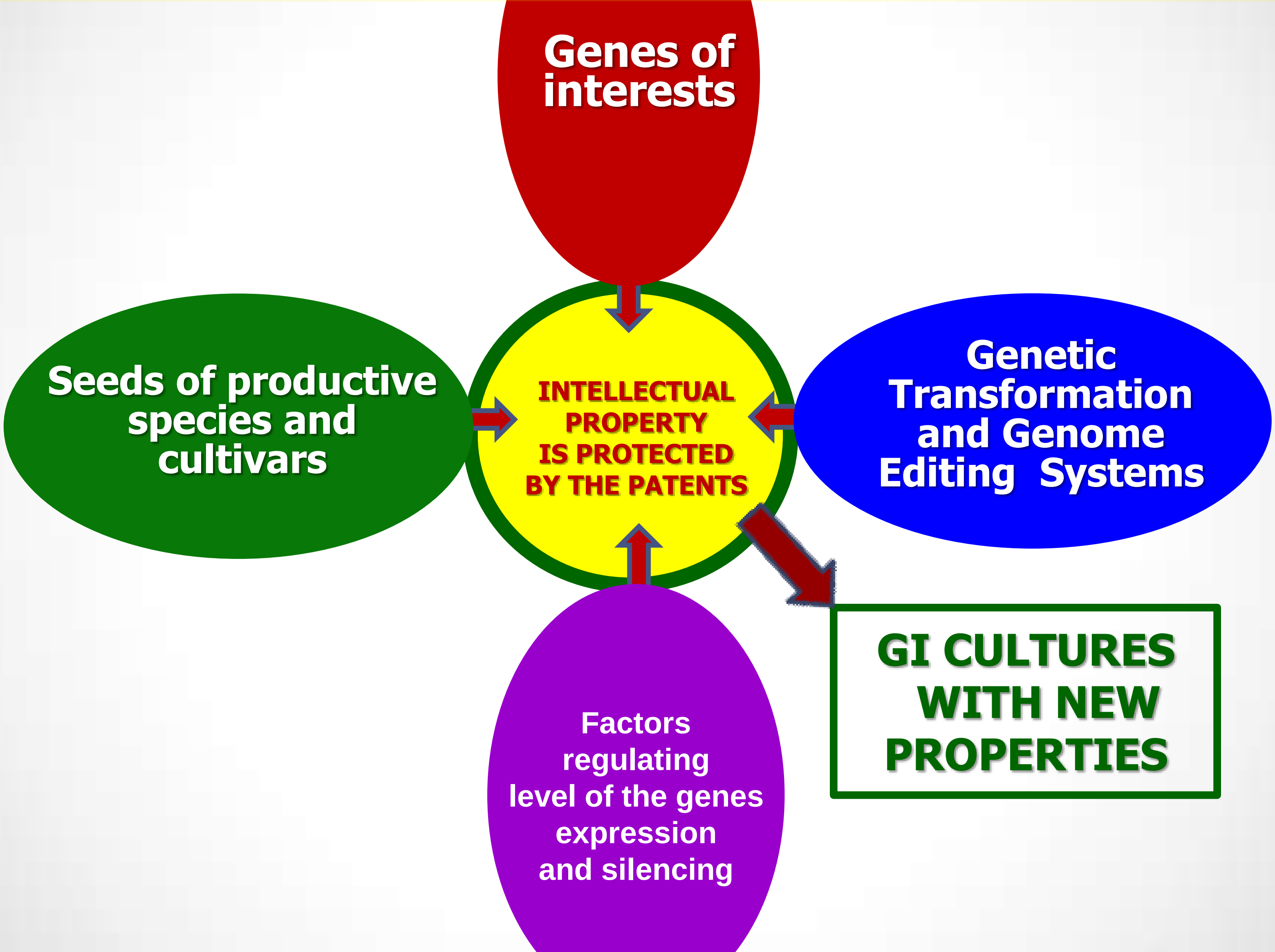
Marker *uidA (GUS)* gene expression in the transgenic sunflower:

A - Inflorescence cut; **B** - flowers with developing seeds;
C - germinating seeds of the T_1 (2nd generation) &
D - different tissues of transgenic plant.



Prof. Alex K. Gaponenko, Koltzov institute of development biology RAS, Moscow

REQUIRED COMPONENTS FOR MODIFYING PLANT METABOLISM BY GENETIC ENGINEERING



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MAT for rubber synthesis genes transfer

MEMORANDUM OF UNDERSTANDING
FOR RESEARCH COLLABORATION BETWEEN
KOREA KUMHO PETROCHEMICAL LTD AND
THE CENTRE "BIOENGINEERING", RUSSIAN ACADEMY OF SCIENSES.

1. Korea Kumho Petrochemical Co., Ltd., Gwangju, Korea (hereafter "KUMHO") and the Centre "Bioengineering", Russian Academy of Sciences (hereafter "CBRAS") agree to collaborate in a project of research activities (hereafter "the Project") related to rubber biosynthesis.
2. The duration of this Memorandum of Understanding shall be one year from the date of this agreement, and is subject to an extension upon the agreement of KUMHO and CBRAS. This agreement may be terminated by KUMHO or CBRAS giving a six-month notice.
3. KUMHO and CBRAS will supply each other with research materials in the form of experimental data and research preparations for the purpose of undertaking the Project. Such materials, either in its original form or propagated or multiplied from its original form shall not be released to another party without the consent of KUMHO and CBRAS in writing.
4. KUMHO and CBRAS shall provide each other with regular verbal or written progress reports on the Project. Such reports shall not be released to another party without the consent of KUMHO and CBRAS in writing, unless the information is already available in the public domain otherwise than by breach of this agreement or is legally received From a third party without obligation of confidence. Consultants of KUMHO and CBRAS are excepted from this condition of confidentiality, but upon receipt of the above mentioned confidential information, KUMHO and CBRAS shall be liable to ensuring that their respective consultants be subject to the same conditions of confidentiality. These obligations of confidentiality shall expire ten years from the date of this agreement.
5. The results of the Project shall be kept confidential by KUMHO and CBRAS and shall not be used or disclosed to third parties unless such disclosure is agreed upon in writing by KUMHO and CBRAS, or unless the information is already available in the public domain otherwise than by breach of this agreement or is legally received from a third party without obligation of confidence. Consultants of KUMHO and CBRAS are excepted from till condition of confidentiality, but upon receipt of the above-mentioned confidential information. KUMHO and CBRAS shall be liable to ensuring that their respective consultants be subject to the same conditions of confidentiality. These obligations of confidentiality shall expire ten years from the date of this agreement.
6. Until a decision is taken to commercialize the results of the Project, KUMHO and CBRAS shall each be responsible for financing the research undertaken in the respective organizations, except where alternative arrangements, agreeable to both parties, are made separately.
7. If the results of the Project are of interest, KUMHO and CBRAS shall consult with a view to making arrangements concerning commercial exploitation. In the event the decicion is taken to commercialize the results of the Project, KUMHO and CBRAS shall share the costs thereafter of developing the commercial product and shall share

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the profits derived from the commercial product. Such costs and profits shall be shared between KUMHO and CBRAS in the event that commercialization of the Project involves essentially research findings obtained as a result of the research collaboration between KUMHO and CBRAS. In the event that the commercialization of the Project involves the application of proprietary information, expertise or materials that are acquired and owned either by KUMHO, or CBRAS, or Dr. Alex Gaponenko prior to the research collaboration KUMHO, CBRAS and Dr. Alex Gaponenko shall consult with a view to arrive at a mutually agreeable sharing of the profits in a manner that reflects the proportional contribution of KUMHO, CBRAS and Dr. Alex Gaponenko to the commercialization of the Project.

8. KUMHO and CBRAS shall jointly apply for patents on patentable inventions with the cost shared between KUMHO and CBRAS. However, if one party does not wish to be included as a joint applicant, the other party may apply for the patent in its own name and meeting the entire cost involved.
9. The any profits of CBRAS in any patents or commercialization of projects results will be shared equally between CBRAS and Dr. A.K.Gaponenko, who is the owner of know-how and Russian Federation Patents for obtaining transgenic sunflower and who will carry out the project. For providing of this condition Dr. A.K.Gaponenko must be include as one of inventors and owners of any patent, which will be issued in a results of this project.
10. Any questions on this record of discussions or any other problem that may arise shall be settled by KUMHO and CBRAS in spirit of goodwill and understanding. In the event, however, that the parties cannot definitively resolve any question or problem ("dispute") relating to this MOU, they agree to have such dispute settled by a panel of three (3) independent arbitrators pursuant to the rules and procedures of the International Chamber of Commerce in Stockholm or Paris. The venue for arbitration shall be the country of the respondent. The arbitrage award shall be final and binding upon the parties. This MOU shall be governed by, and construed in accordance with the laws of the Sweden, without regard to its conflict-of-laws provisions.

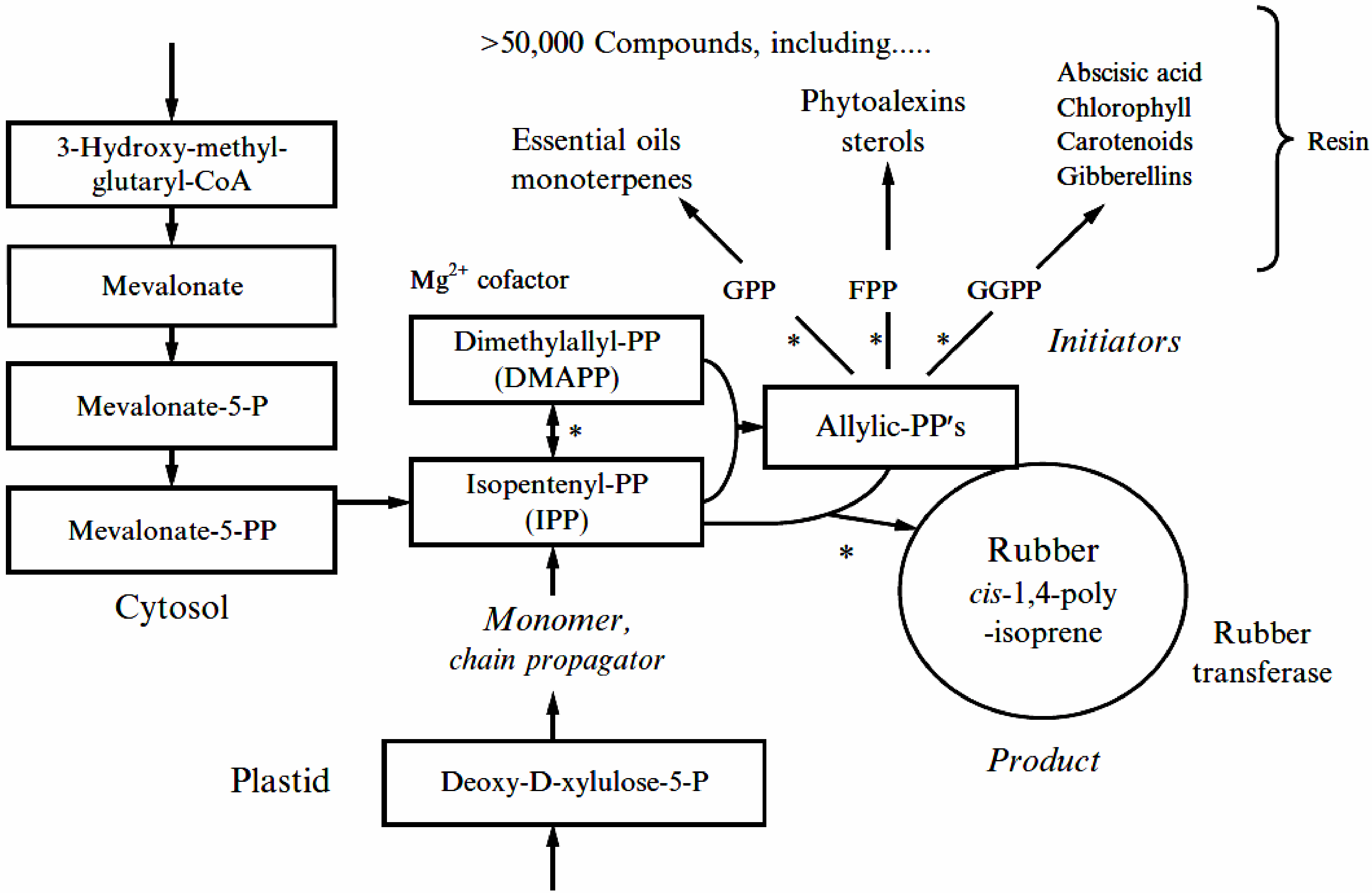
Signed:
Name: Pill-Soon-Song
Designation: Vice President
For Korea Kumho Petrochemical Co
Date:

Signed:
Name: Konstantin Skryabin
Designation: Director of the Centre Bioengineering, Russian Academy of Sciences
For The Centre "Bioengineering" RAS

Signed:
Name: Alex Gaponenko
Designation: Head of the Plant Cell Engineering Laboratory of The Centre Bioengineering, Russian Academy of Sciences
Date:

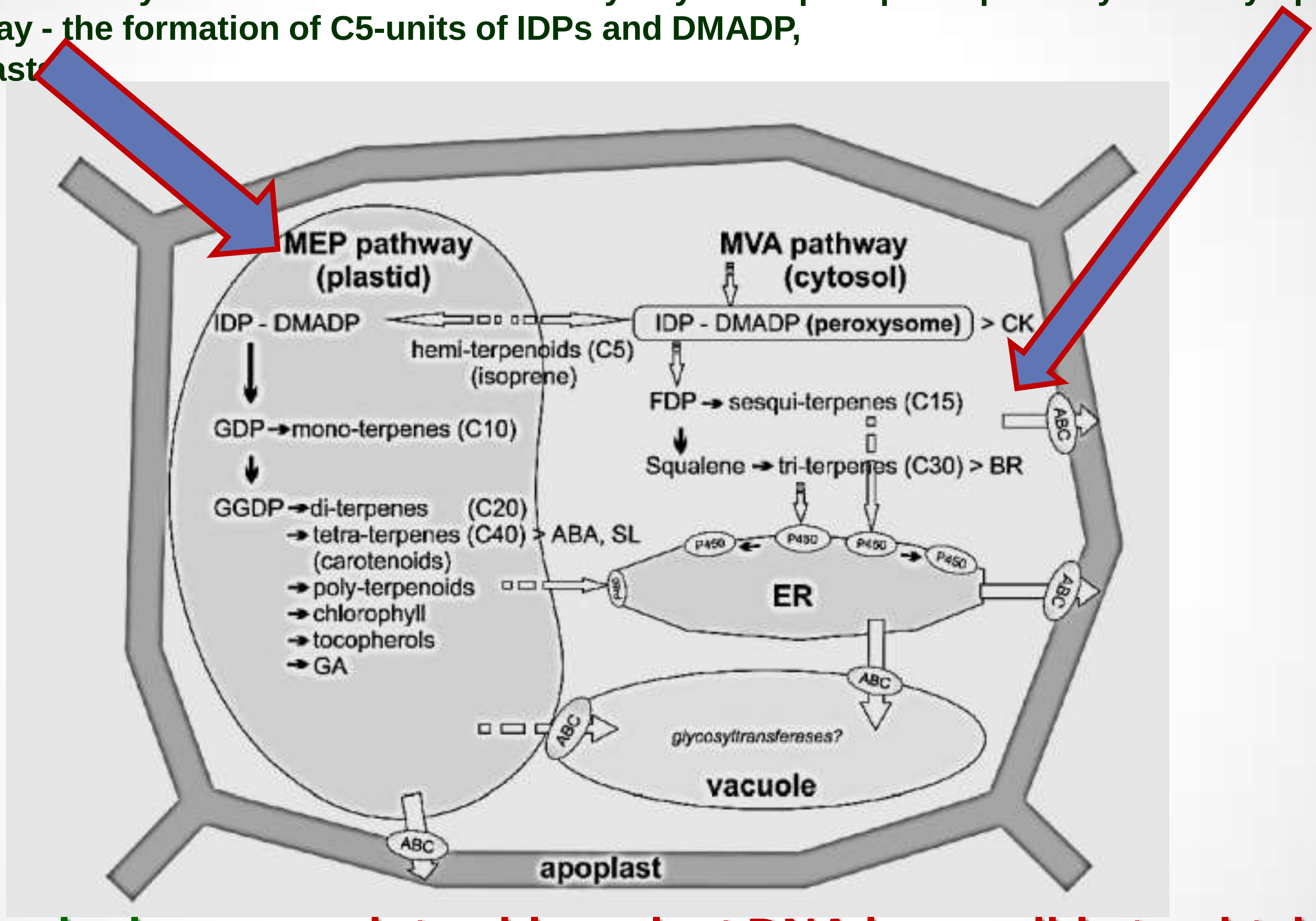
Cpt-1	Cis-prenyl transferase	*Hevea brasiliensis*
PPI	Isopentenyl pyrophosphate synthase	*Hevea brasiliensis*
FPS	Farnesyl pyrophosphate synthase	*Helianthus anuus*
GGPS	Geranyl geranyl pyrophosphate synthase	*Helianthus anuus*
HMGR	3-Hydroxi 3-methylglutaryl-CoA reductase	Arabidopsis thaliana

Figure 4.1 The isoprenoid pathway, including the enzymes that can also compete with rubber transferase for isopentenyl pyrophosphate (monomer), allylic pyrophosphate (initiator), and magnesium ions (activator).



To modification of metabolic pathways in plants. Compartmentation isoprenes biosynthesis in a plant cell.

Two independent ways: the mevalonate and methyl erythritol phosphate pathway in the cytoplasm, MEP pathway - the formation of C5-units of IDPs and DMADP, in chloroplasts



UNDER introducing genes into chloroplast DNA is possible to obtain up to 46% of the new protein or polymer, the amount of soluble total protein in cells by nuclear transformation is not more than 2.5%

For obtaining high level of transgene expression and avoid gene flow into the environment we need to develop:

SUNFLOWER CHLOROPLAST GENETIC TRANSFORMATION.

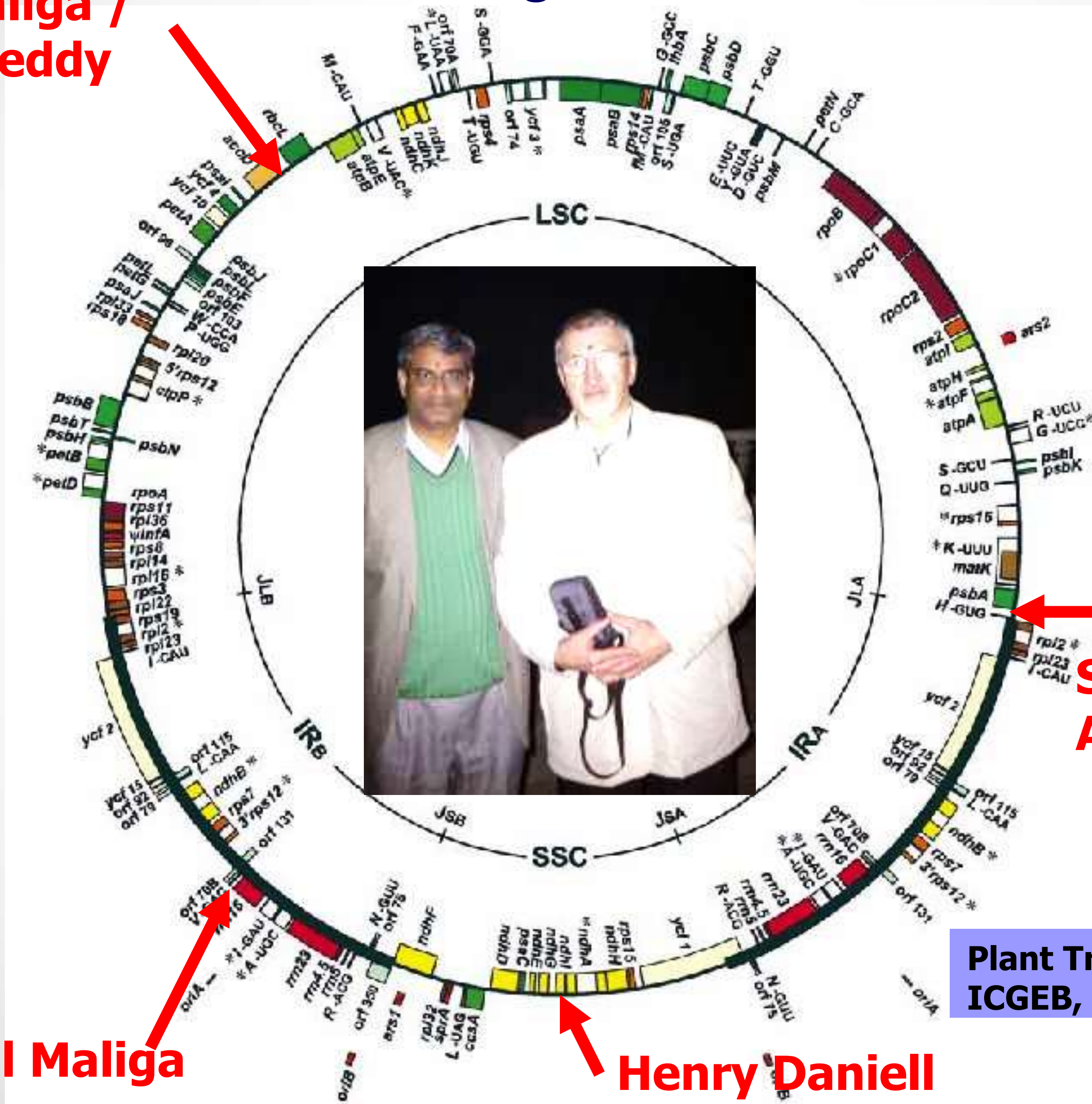
Comparison of the chloroplast & nuclear genome transformation features

Transgenic	Chloroplast genome	Nuclear genome
Transgene copy number	Up to 10000 copies per cell	A few copies per cell
Level of gene expression	Polyploidy & some other features results in the expression level of transgene up to 46% of TSP*	Gene regulation determines the rate of transcription expression level usually less than 3-5% of TSP*
Gene arrangement and transcription	Genes arranged in operones, mRNA is polycistronic; multiple transgenes can be introduced in a single transformation event	mRNA is monocistronic, each transgene is inserted independently
Gene silencing	Not reported	Transcriptional and post-transcriptional silencing occurs
Gene containment	Maternal gene inheritance in most crop plants prevent gene flow to the environment	Paternal transgene inheritance results in out-crossing among crops and weeds

*TSP - total soluble protein

Sites of genes integration in the sunflower plastid DNA *via* homologous recombination

Pal Maliga / Siva Reddy



Siva Reddy/ Alex Gaponenko

Pal Maliga

Henry Daniell

Plant Transformation Group
ICGEB, New-Delhi

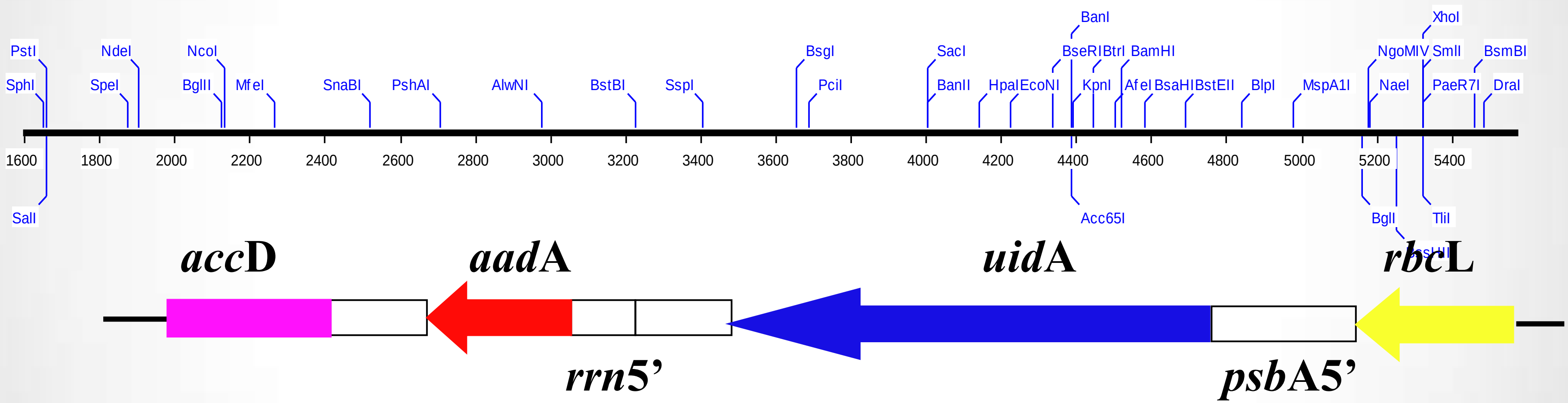
Exchange visits

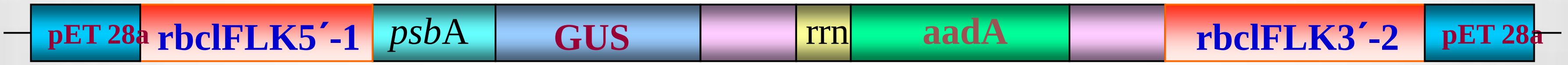
Prof. Alex Goponeko visited ICGEB from Russian side discussed the progress of the work and he also presented a talk providing their side progress

Materials exchanged

Chloroplast transformation vectors that are suitable for sunflower were prepared and provided to Russian collaborators

pVSR326





pET28arbclFLKsunVSR (11293bp)

Prof. Alex K. Gaponenko, Koltzov institute of development biology RAS, Moscow

Main steps for the development of sunflower chloroplast transformation technology (Indo-Russian Mutual Project).

- Development of leaf and immature embryos-based plant regeneration systems - √ DONE
- Optimization of the biolistic parameters for gene delivery into cell organelles - √ DONE
- Sequencing of some sunflower chloroplast DNA regions for vector development - √ DONE
- Development of sunflower - specific plastid vector for homologous recombination mode of transgene integration in the chloroplast DNA -√ DONE
- Development of the selection procedure for obtaining the uniform - homoplastomic nature of transformed plants - **WE NEED FINANCIAL PROVISION**

Agronomic and natural rubber characteristics of sunflower as a rubber-producing plant

Calvin H. Pearson[a], Katrina Cornish[b], Colleen M. Mcmahan[b], Donna J. Ratha, Jenny L. Brichta[b], Jennifer E.Van fleet A Colorado State University, Agricultural Experiment Station, USA; b USDA-ARS, Western Regional Research Center, Albany, California, USA

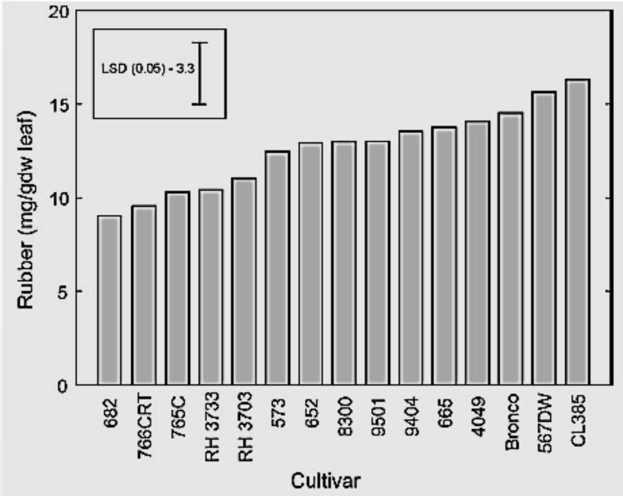
1. SUNFLOWER SYNTHESIZES 1 - 1.6% OF NATURAL RUBBER (NR) by dry weight of the leaves.

2. SUNFLOWER SYNTHESIZED NR with 95–97% being low molecular weights ranging from 66,000 to 74,000 g/mol and a small, remarkable percentage (~5%) of NR being higher molecular weight (~600,000 g/mol).

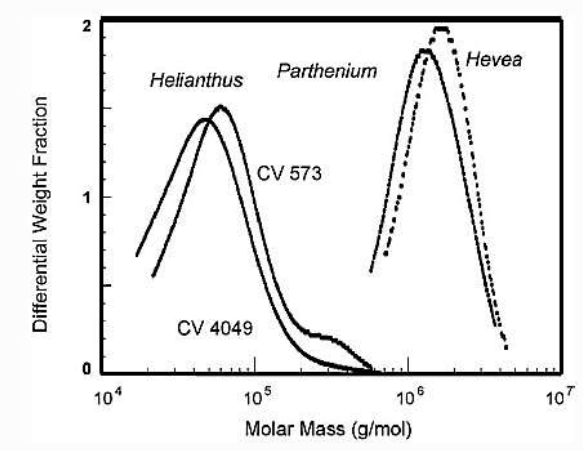
3. RUBBER PARTICLE SIZE ANALYSIS: Heavy and light NR particles, as found in sunflower also are found in *Ficus elastica. F. elastica* and *H. annuus* (HA372 and Hopi), both have particles larger than *H. brasiliensis* and *P. argentatum,* and both make significant amounts of low molecular weight NR

4. THE POTENTIAL FOR INCREASING LATEX PRODUCTION IN SUNFLOWER APPEARS POSSIBLE, given current levels are low and reasonable advances in latex production in sunflower plants through plant breeding and genetic engineering could be achieved.

5. THE DEVELOPMENT OF SUNFLOWER CULTIVARS SUITABLE FOR COMMERCIAL PRODUCTION OF NR will require significant improvements in the quantity and quality of NR produced in the plant.



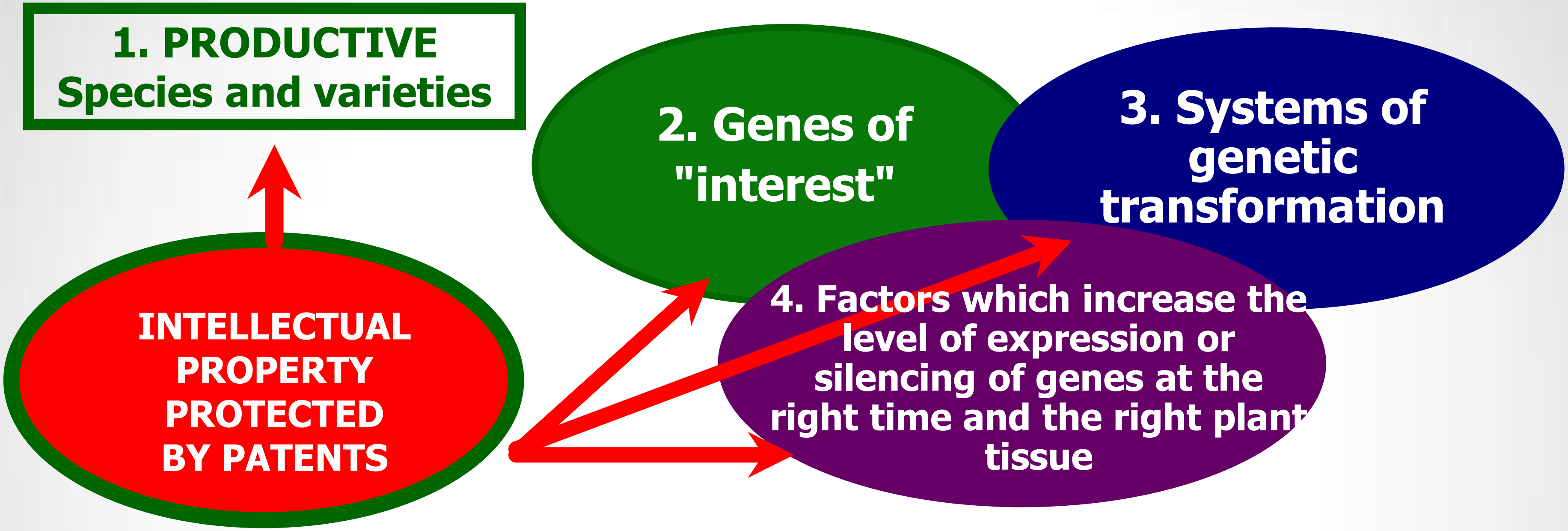
Natural rubber that was solvent extracted from leaves of sunflower cultivars grown at Fruita, Colorado during 2002.



Molecular weight profile of latex rubber purified from two sunflower cultivars grown in Colorado compared with latex rubber purified from *Hevea brasiliensis* and *Parthenium argentatum*.

Source: Industrial Crops and Products 31 (2010) 481–491

REQUIRED COMPONENTS FOR MODIFYING PLANT METABOLISM BY GENETIC ENGINEERING



CULTURE WITH IMPROVED PROPERTIES

The ideology of the project	Developed
Experience in the implementation of projects of creation of GM crops	There Is In Stock
Seeds of rubber species	There Is In Stock
Genes of Rubber biosynthesis	Part Of The Genes In Stock
Genetic Transformation System	Possess And Have The Experience Of Creating
Qualified personnel	There Is In Stock
Equipment, greenhouse	There Is In Stock

OBJECTIVE OF THE PROJECT:

Creating a domestic alternative crops, producing high quality rubber for the rubber industry

PROJECT OBJECTIVES:

I. Development the system of the sunflower chloroplast genetic transformation of to increase DNA synthesis and nuclear genetic transformation of Jerusalem artichoke for the improvement of their natural rubber properties.

II. The identification and isolation of genes of rubber biosynthesis* for genetic modification for sunflower or Jerusalem artichoke:

A. Increasing the molecular weight of the Sunflower and Jerusalem artichoke Natural Rubber

B. Growth of plant productivity and improve the properties of NR

III. Editing sunflower genome biosynthesis genes NC Brazilian rubber trees to improve the properties of natural rubber sunflower.

IV. Transformation of chloroplasts of sunflower to increase synthesis isoprenes

V. Assessment of the productivity of sunflower and Jerusalem artichoke rubber

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*Implemented and carried out by our former partners in the project: Kumho laboratories, Korea Kumho Petrochemical Co., Ltd. Ulsan, Korea. Some genes involved in the biosynthesis of natural rubber, were provided to us are:

Cpt-1 - *C*is-prenyl transferase из *Hevea brasiliensis*
PPI - Isopentenyl pyrophosphate synthase из *Hevea brasiliensis*
FPS - Farnesyl pyrophosphate synthase из *Helianthus anuus*
GGPS - Geranyl geranyl pyrophosphate synthase из *Helianthus anuus*
HMGR - 3-Hydroxi 3-methylglutaryl-CoA reductase из Arabidopsis thaliana

Under the leadership of A.K. Gaponenko successfully completed projects

	Project name	The amount of financing	Source of financing	Duration	The main results obtained
1	The development of the evaluation system prediction of negative consequences for human health associated with the consumption of products derived from the use of genetically modified organisms or containing genetically modified organisms.	4,8 million rubles	The Ministry of Education and Science of the RF Civil Code № 2014-14-576-0160.	2014	The regulations rules have been created
2	Isolation of proteins, insecticidal for insect of the Hemiptera genus	1.3 million rubles	Pioneer Hi-Bred International, Inc., USA.	May - November 2012.	It has been shown the insecticidal action of BT proteins on artificial diet with BT proteins for bug
3	The development of biotechnology and the industrial production of seed of genetically modified agricultural. cultures "Establishment of sugar beet resistant to herbicides and viruses.	150 million rubles (5 millions of US dollars)	Ministry of Industry, Science and Technology of the RF, Civil Code № 02.190.006	11.03.2003 - 3.12.2006	The RF patent for method of genetic transformation of sugar beet has been obtained. Create lines of sugar beet resistant to the herbicide Basta. The limited field trials of GM sugar beet lines have been conducted.
4	Creating forms of transgenic wheat with increased tolerance to drought and salinity.	0.5 million rubles	Ministry of Industry, Science and Technology of RF	2004	The lines of transgenic wheat expressing *GST* gene resistant to NaCl salinity were obtained.
5	**Development of genetic transformation of sunflower chloroplast to prevent genes leakage into the environment and the high expression of transgenes**.	0.9 million rubles	Grant CRP-02, ICGEB, New Delhi, India	2006-2009	**A system for induction of multiple apexes, include the system of selection of transgenic tissues. Work was stopped due to the transition of Gaponenko to another institute**
6	Create sunflower resistant to broad-spectrum herbicide Basta. 2008	0.5 million rubles	Presidium RAS, "Support for Innovation"	2008	Established cell lines of transgenic sunflower e expression bar gene, resistant to the herbicide Basta
7	Development of sunflower regeneration and genetic transformation systems	$60 000	Rhone-Poulenc, Biotechnology Center of Ohio State University, Lab. of Professor John Finer.Wooster, USA,	1993 -1994	Sunflower genetic transformation systems have been established
8	Molecular control of the formation and development of inflorescence of sunflower	$60 000	(NOW) - the Netherlands Organisation for Scientific Research. Centre for Plant Breeding and Reproduction Research (CPRO-DLO), Vagengen	1995 -1998	HAM MADS genes of sunflower have been isolated. Transgenic sunflower lines expressed HAM genes were obtained and studied

Thank you for attention,
Alex Gaponenko