UDC 631.147:633.85

AGROBACTERIAL TRANSFORMATION OF SPRING RAPE WITHOUT IN VITRO REGENERATION PHASE

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The results of approbation of acquiring forms of spring rape resistant to the herbicide of continuous action with the active substance phosphenotritsin are given.

Spring rape is successfully cultivated in areas of risky growing of winter rape. It is a good insurance culture. In unfavorable years during the winterkilling of winter rape, the areas can be sown with spring rape. To obtain high yields of spring rape, effective remedies of combating with weeds are required. The use of herbicides of continuous action almost completely solves this problem.

Since the mid-1990s, an American company Monsanto and a German company BayerCropScience created a genetically modified rape resistant to glyphosate herbicide (Monsanto) and gluphosinate (BayerCropScience). At present, there are many varieties and hybrids of genetically modified plants: soybean, corn, alfalfa, beets and others [2].

To create transgenic plants resistant to herbicide, the natural transformation system Ti-plasmid (from English *Tumor inducing plasmid)* of soil agrobacteria *Agrobacterium tumefaciens* is used [1].Unique biological properties Ti-plasmids make it an ideal natural vector for gene transfer. Ti-plasmid has a wide range of hosts, it embeds the T-DNA (from English *Transforming DNA*) into plant chromosomes, where it can be transmitted and its genes are translated with proteins formation. Borders of the T-DNA are defined by direct sequences which are repeated by length of 25 nucleotide pairs, any piece of alien DNA inserted between repetitions will be transferred to plant cell. However, manipulation with the Ti-plasmid is complicated due to a large size, to insert a gene into plasmid by a traditional way is impossible. Therefore, the Ti-plasmid was modified by genetic engineering methods, and on its basis, vectors for the transformation of plants were obtained [3].

A vector should contain the gene sequence that should be entered into the genome of plants and to be under the control of the promoter which is capable to express in plant cells. Except the functional genes, the vector should have marker genes of transformation. Genes, resistant to antibiotics and herbicides, are used as markers [6].

Alien genes in rape plants can be implemented by direct methods (biobalistic, electroporation, microinjection and others). However, most of the works on the creation of herbicide-resistant rape is performed by different methods using agrobacteria [3]. The effectiveness of such transformation depends on choice of recipient plants, that is why there is no universal method of agrobacterium mediated transformation of rape, and the development of methods for transformation remains relevant.

In 1993, Bechtold with coauthors developed a genetic engineering method of

vacuum infiltration of plant explants in agrobacterium mediated suspension. Based on this, they invented a method of transformation using agrobacteria in the field, in other words, on flowering plants growing in the open soil. This method is called *inplanta* (from English on plants) [7].

The purpose of the research is the aprobation of the method of agrobacterium mediated transformation *in planta* on spring rape, and the possibility of obtaining herbicide-resistant forms of the active substance phosphenotritsin (commercial name “Basta”).

Methods of the research. The research was conducted under the laboratories and field conditions of Department of Genetics, Plant Breeding and Biotechnology of Uman National University of Horticulture during 2010-2012.

For transformation were used *Agrobacterium tuimfaciens* with a plasmid containing the *bar-gene (bialaphos resistance)*, which determines resistance to phosphenotritsin, an active substance of bialafos herbicide – "Basta". A plasmid includes marker genes of resistance to antibiotics and is placed under 35SCaMV promoter of cauliflower mosaic virus [4].

As a recipient of spring rape Dobrobut, Aydar and VNIS 100 varieties were used.

Agrobakteria have been cultivated for two days on LB nutrient medium with antibiotics added. The cultivation was performed on the rocker (150 rev/min) in the dark at the temperature of 28 C°. After the increase of bacteria, sucrose and surfactant *Silwet L-77* were added, which facilitates the penetration of bacteria into the intercellular environment [5].

Rape inflorescences were isolated by parchment insulators till flowers bloom. After blooming of most flowers, the plants were treated with agrobacterium mediated cell suspension in the field. Inoculation was performed by dipping the flowers in the bacterial suspension with mild shaking to facilitate the penetration of bacteria into flowers cells. Inoculation time was 1 min. After inoculation, the plants were covered with plastic bags irrigated with the water for 24 hours to create conditions of high humidity. Then rape was isolated with parchment insulators and was grown to obtain seeds.

Results of the research. 67 plants of Aidar variety, 58 plants of VNIS 100 variety and 85 plants of Dobrobut variety were treated with bacterial suspension. Seeds collected from mentioned plants were systematized and sown into the soil in spring. Young growth of spring rape were obtained in the following quantities: Aidar variety – 870 plants, VNIS 100 variety – 631 plants, Dobrobut variety – 1251 plant. Selection of phosphenotritsin-resistant forms was made by spraying plnts with solution of the "Basta" in the development phase of 4-5 pairs of leaves. Herbicide dose was 7 ml per liter of water.

On the fourth day after spraying with herbicide, leaves of the majority of plants began to turn yellow, changed the color to almost white, began to dry and they died. In all died 865 plants of Aidar variety, 638 plants of VNIS 100 variety, 1248 plants of Dobrobut variety. These forms of rape are not resistant to the herbicide. Several plants after spraying herbicide had a green color and continued to form vegetative organs. This indicates that the forms are resistant to the herbicide (Table 1).

1. Frequency of transformation of spring rape forms received by the method of *in planta* in 2011

Rape varieties	Plants		Received transformants	
	In total	Lost	pcs.	%
Aidar	870	865	5	0.6
VNIS 100	638	631	7	1.1
Dobrobut	1251	1242	9	0.7

On the following year, rape seed which survived after the herbicide cultivation was sown into the soil. After the appearance of 4-5 leaves, the herbicide cultivation was held under the same scheme as the previous year. The results are shown in table 2.

2. Inheritance of phosphenotritsin-resistance for 2012

Rape varieties	Plants in total					
	before the cultivation		lost		resistant	
	pcs.	%	pcs.	%	pcs.	%
Aidar	86	100	1	1.1	85	98.8
VNIS 100	97	100	-	-	97	100.0
Dobrobut	150	100	3	1.9	147	97.9

To study the inheritance and expression of genetically modified feature, we have obtained the form of spring rape as a result of self-pollination. Most plants maintained resistance to herbicide, gene expression indicates possible incorporation of the *bar* gene and its inheritance.

It was also found that transgenic plants did not differ phenotypically from normal plants (non-transgenic) of spring rape. Introduced into the genome of plant the *bar* construction has no effect on the expression of functional and structural genes of plants.

Conclusions. After inoculation into agrobacterium mediated suspension of spring rape flowers by the method *in planta,* we have obtained the seed that gave forms resistant to the Basta herbicide: 5 plants of Aidar variety, 7 plants of VNIS 100 variety, 9 plants of Dobrobut variety. As a result of self-pollination of these plants the seeds were harvested which was sown into the soil, and after young sprouts appearance they were sprayed with Basta. We obtained forms of spring rape that kept phosphenotritsin-resistance: 85 plants of Aidar variety, 97 plants of VNIS 100 variety, 147 plants of Dobrobut variety. It suggests that the *bar* gene implemented into the genome of plants is inherited and expressed.

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Одержано 26.04.13

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Агробактериальная трансформация рапса ярового без этапа регенерации in vitro

Проведена трансформация ярового рапса с помощью почвенных бактерий Agrobacterium tuimfaciensметодом inplanta. Как известно агробактерии способны заражать двудольные и некоторое однодольные растения и вызывать при этом образования специфических опухолей—корончатых галлов. В связи с этими особенностями, агробактерии начали использовать в генетической инженерии в качестве вектора для переноса чужеродной ДНК. Для создания такого вектора генно-инженерными методами из нее вырезали участки кодирующие опухолеобрвзование и с помощью Ecoli встроили заданые гены. Не мение важным есть происхождение промотора для обеспечивания экспресии встроенного трансгена.

Для трансформации ярового рапса использовали Agrobacterium tuimfaciens с плазмидой, которая содержит ген-bar (bialaphos *resistance*), *что определяет устойчивость к гербициду "Basta" действующим веществом которого является фосфинотрицин. Также плазмида несет маркерне гены устойчивости к антибиотикам и поставлена под промотор 35SCaMV вируса мозаики цветной капусты.*

В качестве растения реципиента были взяты сорта ярового рапса: Айдар, ВНИС100 и Добробут.

Производилось наращивание агробактерий и последующая обработка цветущих растений рапса в полевых условиях. Полученные семена с обработанных бактериями растений весною высеяли в грунт и произвели отбор устойчивых форм путем опрыскивания гербицидом с действующим веществом фосфинотрицин. Получены резистентные формы: 5 растений сорта Айдар, 7 растений сорта ВНИС100, 9 растений сорта Добробут. Данные растения накрыли изоляторами для самоопыления и получения семян, которые высеяны в грунт весной. После появления всходов была произведена обработка растений ярового рапса гербицидом. Полученные формы сохраняли устойчивость к гербициду. Почти все растения данных сортов резистентны к фосфинотрицину.

Существенным преимуществом данного метода является отсутствие этапа регенерации in vitro. А также его легкость в использовании и не большие финансовые затраты.

***Ключевые слова**: агробактериальная трансформация, яровой рапс, Т-ДНК, inplanta.*

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Agrobacterial transformation of spring rape without phase of regeneration in vitro

Transformation of spring rape with the help of soil bacterium Agrobacterium tuimfaciens by inplanta method is conducted. It's generally known that Agrobacterium is capable to infect dicotyledons and some of monocotyledonous plants and to cause formations of specific tumours —crowns galls. In connection with these features, agrobacterium strated to use in the genetic engineering as a vector for the transfer of heterogenous DNA. To create such vector with the help of genic-engineering methods, was cut out from it the areas encoding formations of tumours and with the help of Ecoli embedded given genes. Becoming numb important is the origin of promoters to provide the expression of built-in transgene.

For transformation of spring rape used Agrobacterium tuimfaciens with plasmids that contains gene-bar (bialaphos resistance), that determines stability to herbicide of "Basta" the operating substance of which is phosphinothricin. Also plasmids carries the marker genes of stability to the antibiotics and put under promoters 35S CaMV of virus of cauliflower mosaic.

As a plant recipient the varieties of a spring rape were taken: Aydar, VNIS 100 and Dobrobut.

Upbuilding of Agrobacterium and subsequent treatment of flowering plants of rape was produced in the field terms. Received seeds with bacterial treatment plants sowed in soil in spring and the selection of steady forms was made by sprinkling herbicides with the operating substance of phosphinothricin. Resistent form received: 5 plants of sort Aydar, 7 plants of sort VNIS 100, 9 plants of sort Dobrobut.These plants was covered with insulators for self-pollination and receiving of seed which is sown in the soil in spring. After appearance of sprouts, the treatment of a spring rape plants with

herbicide was made. The received forms saved stability to herbicide. Almost all plants of these varieties are resistant to phosphinothricin.

Substantial advantage of this method is the absence of the stage of regeneration in vitro. And also its easiness in use and small expenses.

***Key words**: agrobacterial transformation, spring rape, T-DNA, inplanta.*