جامعة الكويت
Kuwait University

Approaches of Gene Editing Tools in saffron production

By: Maryam Al-Shiha
Course : Research Topics and seminar I
Advisor : Hasan Al-Hadad

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Outline

- The selected paper

Outline

- The selected paper
- Background about saffron production

Outline

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- Paper objective

Outline

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- Paper objective
- Methodology

Outline

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- Background about saffron production
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- Methodology
- Result and discussion

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- Methodology
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The selected paper

Chib *et al. Plant Methods* (2020) 16:47
https://doi.org/10.1186/s13007-020-00589-2

Plant Methods

The title of selected paper is

RESEARCH Open Access

Development of a system for efficient callus production, somatic embryogenesis and gene editing using CRISPR/Cas9 in Saffron (*Crocus sativus* L.)

Sudha Chib[1], Arulprakash Thangaraj[2], Sanjana Kaul[1], Manoj Kumar Dhar[1*] and Tanushri Kaul[2*]

Abstract

Background: *Crocus sativus* is a recalcitrant plant for genetic transformation and genetic improvement, largely due to difficulties in *Agrobacterium* mediated transformation and vegetative reproduction. Effective genome editing requires proficient callus production and an efficient method to deliver Cas9 and sgRNAs into the plant. Here, we demonstrate *Agrobacterium*-mediated transformation of saffron. Further, we developed a CRISPR-Cas9 based system in this plant, for efficient gene knockout or edits in future.

Results: Efficient callus production and regeneration confers important benefits in developing competent transformation system in plants. More than 70% multiplication rate of callus initiation was achieved from corm slices of saffron subjected to a two-step sterilization procedure and grown on complete MS medium supplemented with 2,4-D (0.5 mg/L), BAP (1 mg/L), IAA (1 mg/L), photoperiod of 16/8 h and 45% relative humidity at 20 ± 2 °C. In vitro cormlet generation was accomplished in 8 weeks by using mature somatic embryos on MS medium supplemented with TDZ (0.5 mg/L) + IAA (1 mg/L) + Activated charcoal (0.1 g/L) at 15 ± 2 °C. The attempt of using *Agrobacterium*-mediated transformation resulted in successful integration of the binary vector into the somatic embryos of saffron with a transformation efficiency of 4%. PCR and Southern blot analysis confirmed the integration of Cas9 into saffron.

Conclusion: The protocol for callus production, somatic embryogenesis and regeneration was standardised. Successful demonstration of integrated Cas9 in this study constitutes first step in developing strategies for genetic manipulation of saffron, which has so far been considered recalcitrant. Furthering the development of this technology holds significant potential for advancing genetic research in saffron by integrating multigene targeting and/or use of recyclable cassettes.

Keywords: Cas9, CRISPR, Gene manipulation, Saffron, Invitro regeneration, Transformation

This fig1.1show The title of the study that chose and show the location of the study (sudha chib etc.,2020)

The selected paper

- The problem is that the previous genetic modification methods are inaccurate and have disadvantages

- Therefore, the researchers in this study focused on developing a technique for transferring the gene to the target location using the developed CRISPR technology.

The selected paper

Abstract

Background: *Crocus sativus* is a recalcitrant plant for genetic transformation and genetic improvement, largely due to difficulties in *Agrobacterium* mediated transformation and vegetative reproduction. Effective genome editing requires proficient callus production and an efficient method to deliver Cas9 and sgRNAs into the plant. Here, we demonstrate *Agrobacterium*-mediated transformation of saffron. Further, we developed a CRISPR-Cas9 based system in this plant, for efficient gene knockout or edits in future.

Results: Efficient callus production and regeneration confers important benefits in developing competent transformation system in plants. More than 70% multiplication rate of callus initiation was achieved from corm slices of saffron subjected to a two-step sterilization procedure and grown on complete MS medium supplemented with 2,4-D (0.5 mg/L), BAP (1 mg/L), IAA (1 mg/L), photoperiod of 16/8 h and 45% relative humidity at 20 ± 2 °C. In vitro cormlet generation was accomplished in 8 weeks by using mature somatic embryos on MS medium supplemented with TDZ (0.5 mg/L) + IAA (1 mg/L) + Activated charcoal (0.1 g/L) at 15 ± 2 °C. The attempt of using *Agrobacterium*-mediated transformation resulted in successful integration of the binary vector into the somatic embryos of saffron with a transformation efficiency of 4%. PCR and Southern blot analysis confirmed the integration of Cas9 into saffron.

Conclusion: The protocol for callus production, somatic embryogenesis and regeneration was standardised. Successful demonstration of integrated Cas9 in this study constitutes first step in developing strategies for genetic manipulation of saffron, which has so far been considered recalcitrant. Furthering the development of this technology holds significant potential for advancing genetic research in saffron by integrating multigene targeting and/or use of recyclable cassettes.

Keywords: Cas9, CRISPR, Gene manipulation, Saffron, Invitro regeneration, Transformation

Fig. 2 show the summary of the sudy (Sudha Chib etc. , 2020)

Background about saffron production

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Background about saffron production

((The comment name))
(Crocus sativus).

- Saffron is called “ red gold “ or “ flower of happiness “ and it is one of oldest and most expensive spices in the world . It is sold by grams as gold. (Barbara Tóth etc , 2019)

Background about saffron production

History…

-Kashmir is one of the oldest historical regions for saffron production, but it faces problems in production.

- Cultivation of saffron requires precision to avoid saffron contamination, as saffron is affected by climatic conditions.

Background about saffron production

traditional agriculture of saffron production

-need suitable and fertile soil mixed with the remains of decomposing animals and plants to raise the soil on the testicles. Maintaining the quality of saffron.

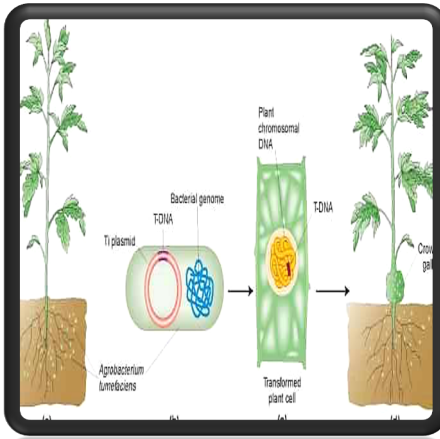
-Saffron can be grown by planting saffron seeds that resemble the shape of onions
In traditional agriculture in an agricultural climate,

saffron is grown in a specific climate under pH 8-7

-the start of the flower and the beginning of the flower may be affected by the climate such as the temperature and the water

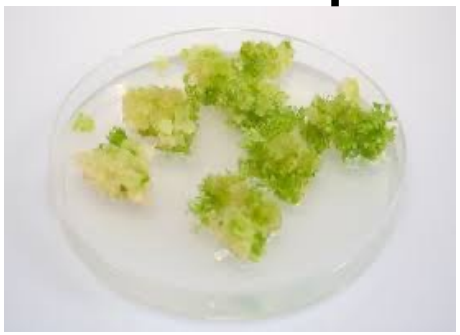
Background about saffron production

-The drawback of the traditional agriculture and the development way



- Reproduction in the traditional way is very slow,
- The tissue culture method was used to produce larger numbers while preserving the genetic trait. It is one of the most important techniques for producing plants that are not infected with viruses. Tissue culture is carried out inside the laboratory in vitro .
- A part of the plants used, and growth regulators are added to it to encourage the formation of the callus, which is regular or irregular undistinguished cells.

Background about saffron production



https://en.wikipedia.org/wiki/Callus_(cell_biology)

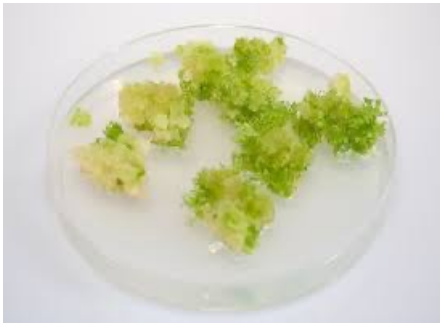
- To improve the production of saffron, the production of callus, which is important to stimulate the plant, must be developed, and it increases the mass of plants

Fig.3 show the location callus of the plant
(..created by maryam alshiha)

Background about saffron production

- **What is the callus ,how produced and what the use of callus ?**

- They are unspecialized cells used in tissue culture - they arise before the roots appear, their color becomes white - at first and after the roots are formed, they become specialized and begin to grow buds that are used in agriculture.
- It is used in the rapid vegetative propagation of selected plants, as the cultivation of callus gives a large number of plants in a short time.
- Callus is often formed due to wounds that occur to the plant parts. It is usually observed on the cut surfaces of roots and stems. Callus consists of plant tissues

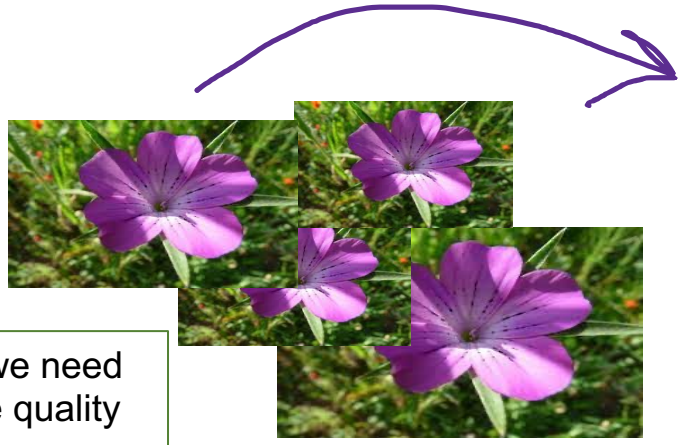


Background about saffron production

question?

Why is saffron considered expensive??

The answer is that it is difficult to harvest.



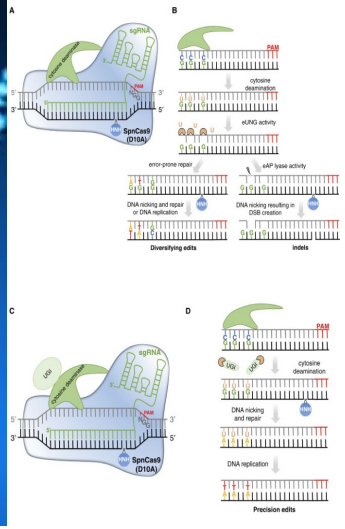
If I want to produce 500 gram of saffron we need to 50 Thuasne of saffron flower. So ,the quality of the production need specific agriculture .

Background about saffron production

- What solutions are available to solve this problem?
- This is the main objective of the study, which is to solve the problem of lack of saffron production due to its impact on environmental f
- Genetically modified plants can used and can be defined as plants to which one or more genes have been introduced by genetic engineering, so the desired traits are transferred by genetic modification without the need for breeding .

Background about saffron production

One of the developed genetic modification methods is the CRISPR tool

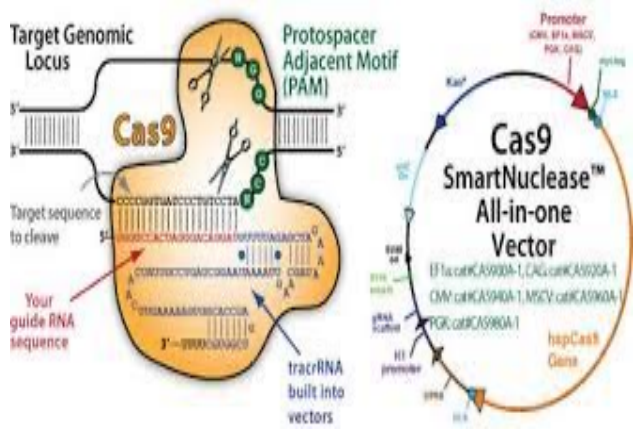


- CRISPR it is cutting, pasting and modifying the genetic genetic sequence of the affected or weak DNA, treating . It has been used to improve agricultural products

- present in bacteria, scientists have benefited from the defense methods that bacteria use to defend themselves...

Background about saffron production

Crisper Cas9 technical requirements

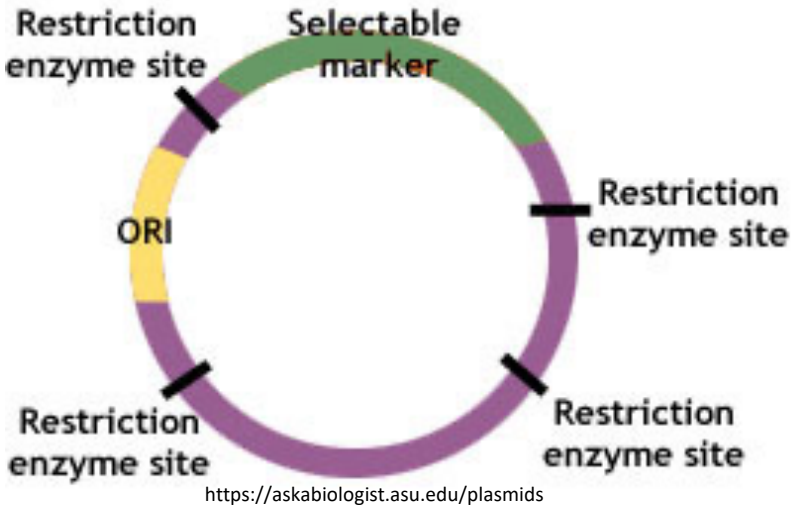


https://www.biocat.com/genomics/genome-engineering/crispr-cas9-smartnuclease-genome-engineering-system-vector-based

- need to design vector for successful gene editing

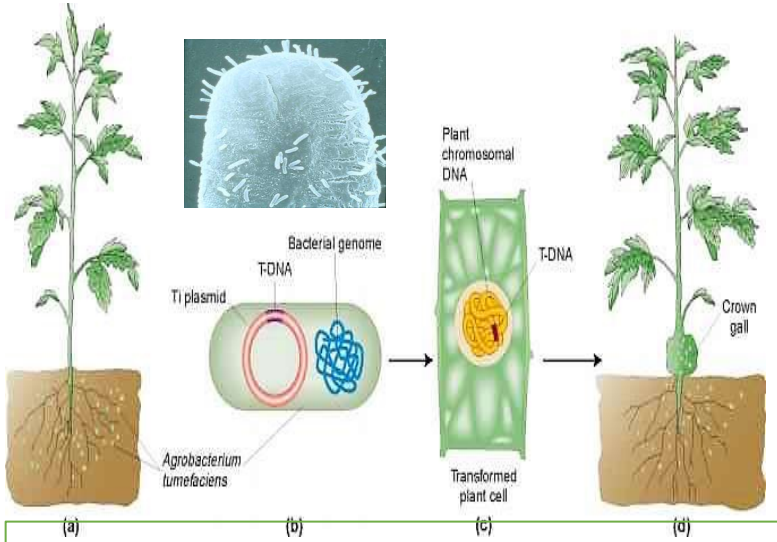
Background about saffron production

transformation and selection step requirement



https://askabiologist.asu.edu/plasmids

Genetic modification needs a vector, using bacteria as a vector of genes, and a plasmid with all the basic characteristics of the plasmid
-origin of replication
-restriction enzyme
-Antibiotic resisting is needed.



Agrobacterium tumefaciens is used as a genetic vector. This bacteria carries the Tumer induced Ti gene in its natural genetic material and infects plants leading to the stimulation of growth hormone.

Paper objective

- The selected paper
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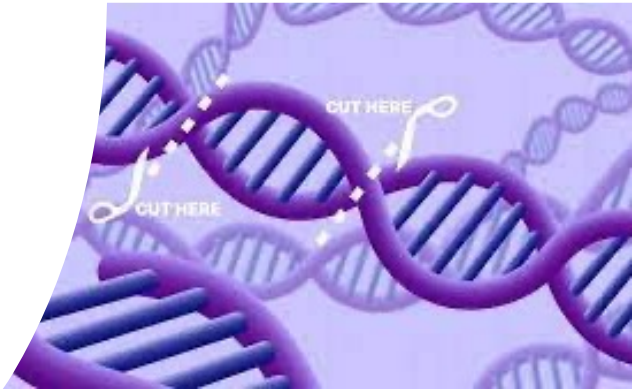
Paper objective

- The aim of the study is to solve the problem of plant impact on the environment, which leads to slow plant reproduction. The study sought to develop callus production and plant physical formation through the CRISPR gene editing tool developed in the laboratory, which leads to plant improvement and bud proliferation (cormalite) and overcoming the traditional method.



Methodology

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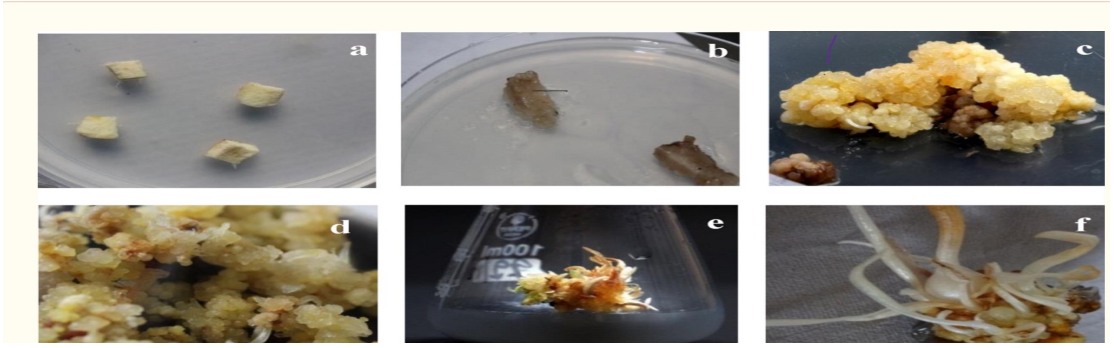
Methodology

- (X plant) Corms was sterilized to remove chemicals under aseptic sterilization
- Then callus produced by inoculated it in MS medium plate with different concentrations of plant growth regulators (BAP,IBA and NAA was added before autoclaving while TDZ was added after autoclaving)
- CRISPR/Cas9 vector was design and (cas9) target gene was inserted to design vector that must have
- -**origin of replication**
  -restriction enzyme
  -Antibiotic resisting
- The design plasmid was transformed using Agrobacterium tumefaciens
- After that, the antibiotic selection media(LB media with kanamycin) was used to culture bacteria on MS (Murashing and Skoog media) that are transformed and used it to select the bacteria that have successfully transformation of cas9 . Which was incubated at 28c for 3 days
- Cefotaxime was added to plant after transformation to observe the resistent of plant after gene editing using CRISPR/Cas9
- PCR and southern blot methods were used to examine the transformation of cas 9 in the plant and count the number of cas9 expression in the plant .

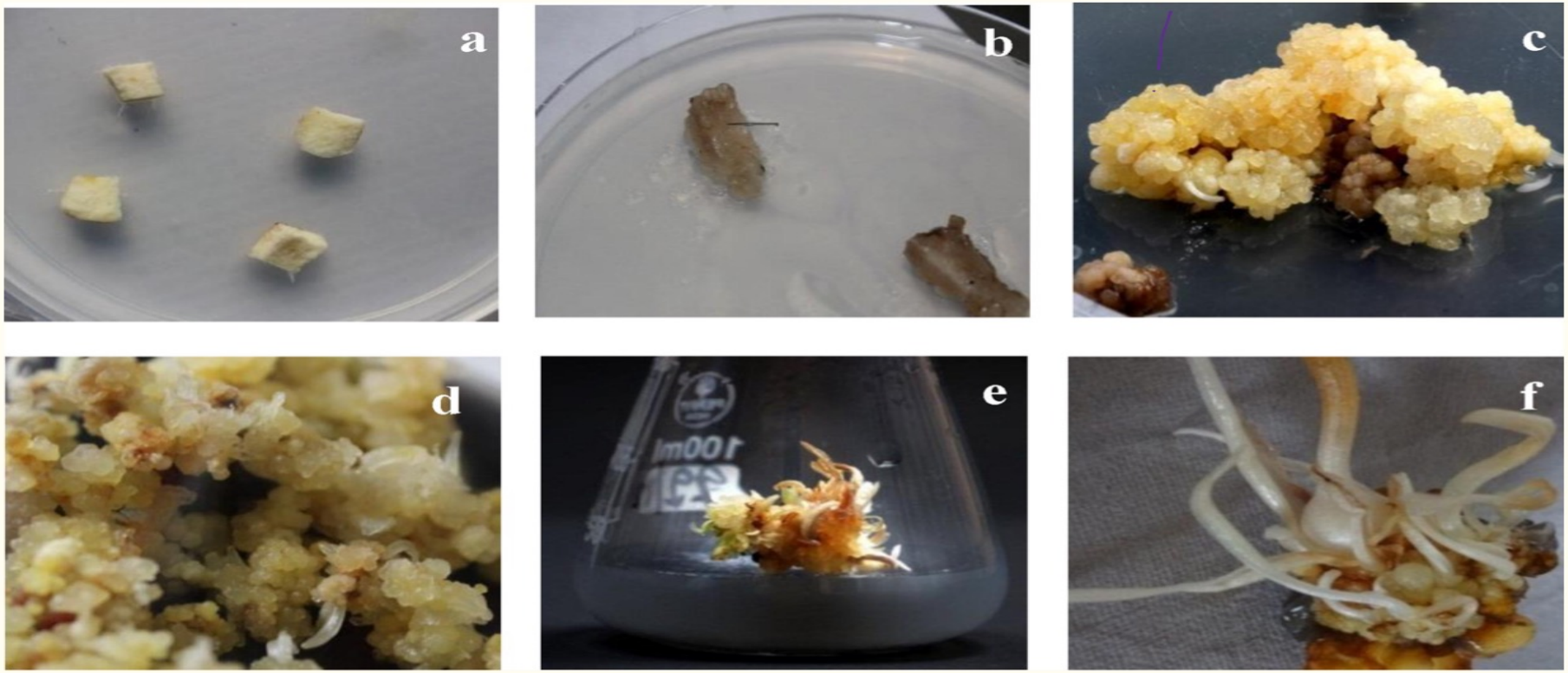


(sudha chib etc.,2020)

Methodology

- callus produced by inoculated it in MS medium plate with different concentrations of plant growth regulators (BAP,IBA and NAA was added before autoclaving while TDZ was added after autoclaving)

Fig 4 show the callus production with addition of plant growth requlators during period



(sudha chib etc.,2020)

Methodology

Sterilization methods

- (X plant) Corms was sterilized to remove chemicals under aseptic sterilization

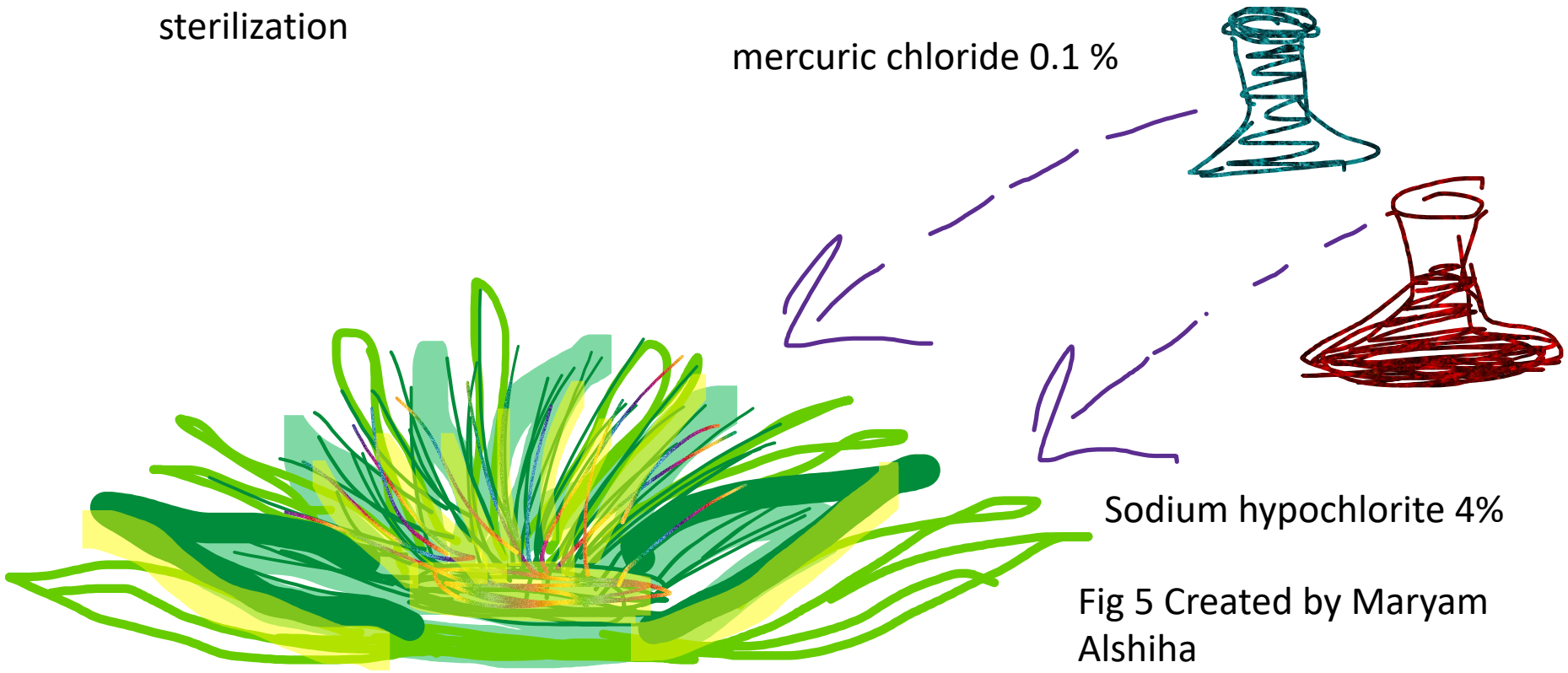


Fig 5 Created by Maryam Alshiha

Methodology

- Then **callus** produced by inoculated it in MS medium plate with different concentrations of plant growth regulators (BAP,IBA and NAA was added before autoclaving while TDZ was added after autoclaving)
- MS (Murashing and Skoog media) media was used

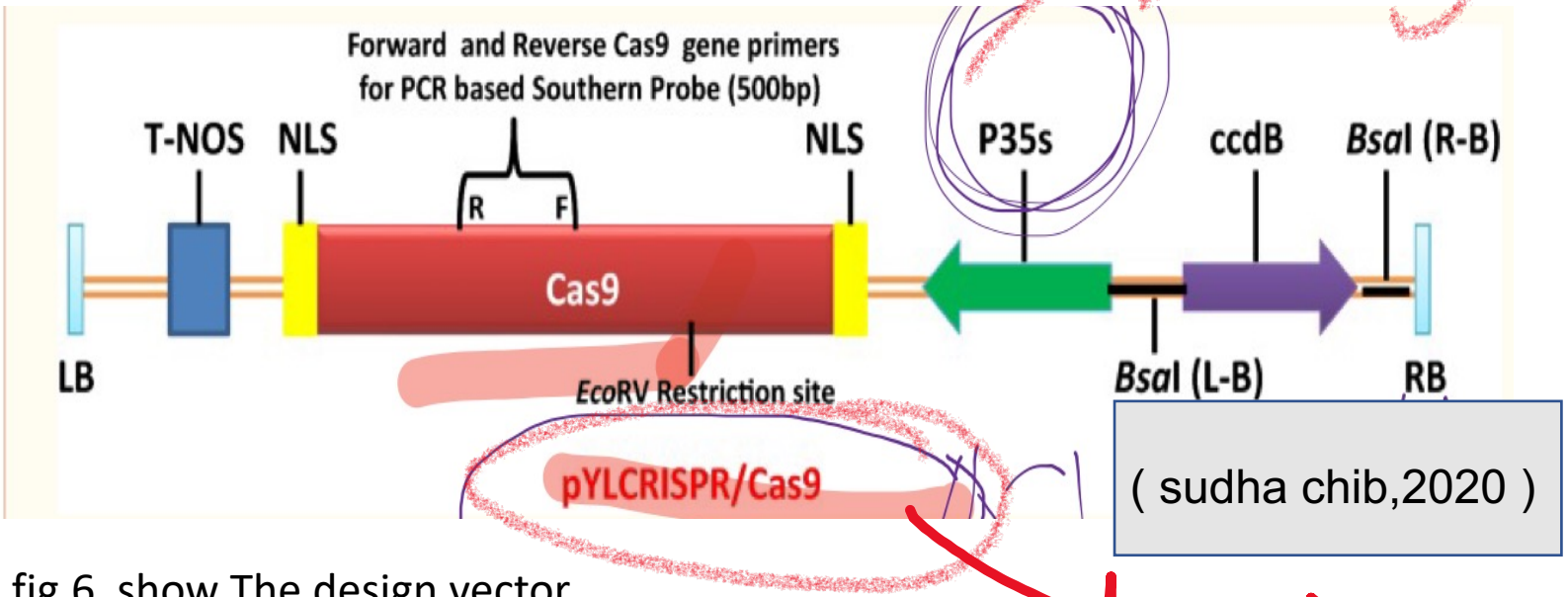
Methodology

CRISPR/Cas9 vector was design and (cas9) target gene was inserted to design vector that must have

-**origin of replication**

-restriction enzyme

-Antibiotic resisting



(sudha chib,2020)

fig.6 show The design vector

From PCAMBIA1300

The modified vector

Methodology

The design plasmid was transformed using Agrobacterium tumefaciens

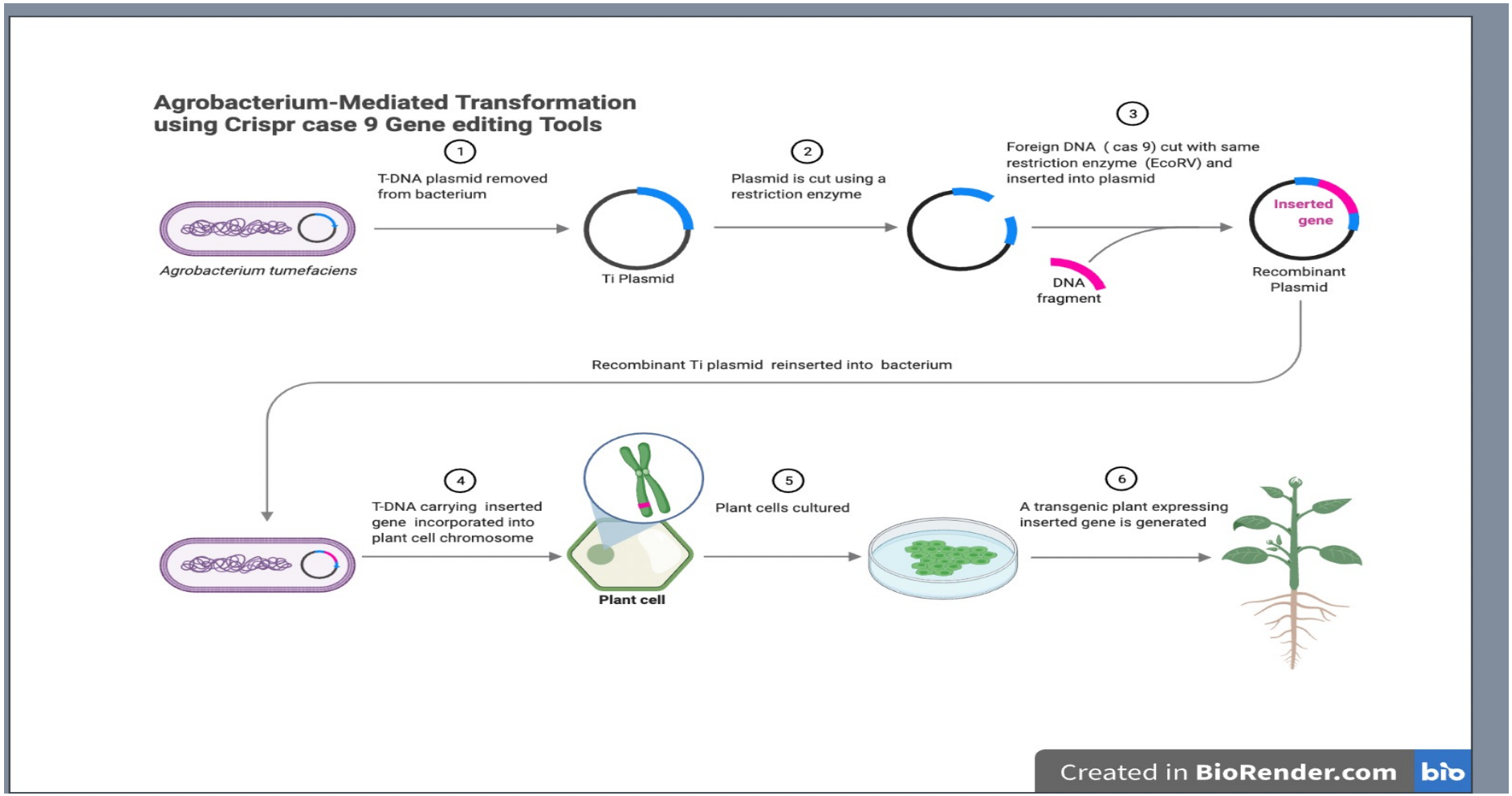
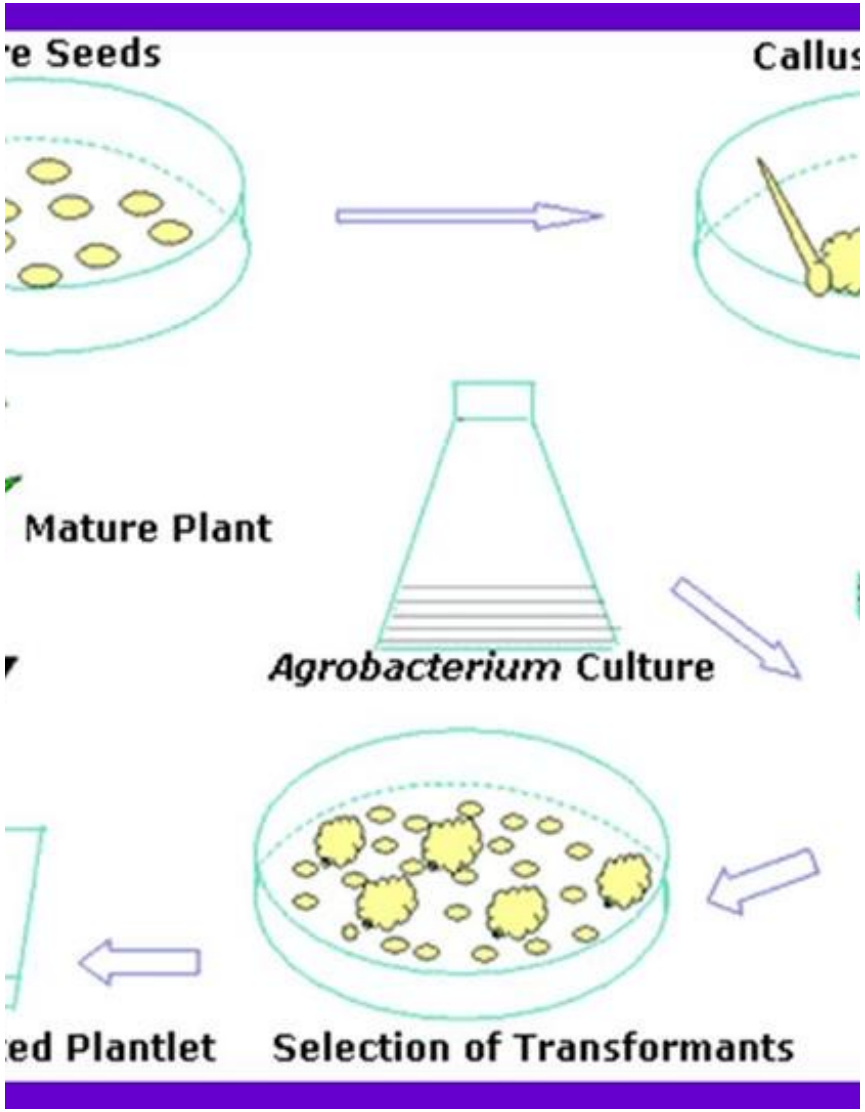


Fig 7 show the experiment step of gene editing tools (modified by maryam alshiha using Biorender.com

Methodology

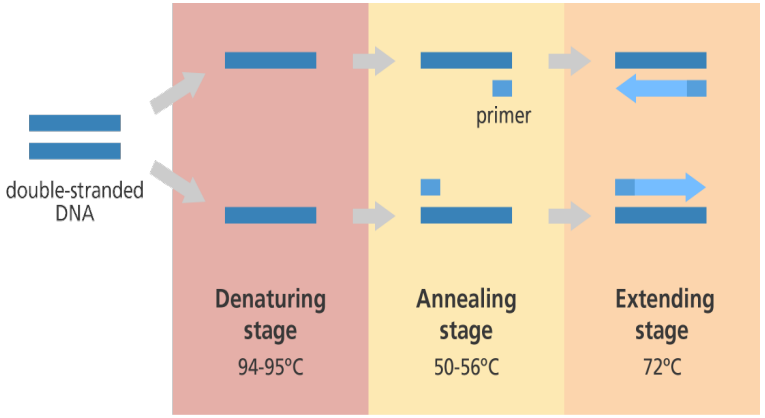
- After that, the antibiotic selection media(LB media with kanamycin) was used to culture bacteria that are transformed and used it to select the bacteria that have successfully transformation of cas9 . Which was incubated at 28c for 3 days
- Cefotaxime was added to plant after transformation to observe the resistant of plant after gene editing using CRISPR/Cas9



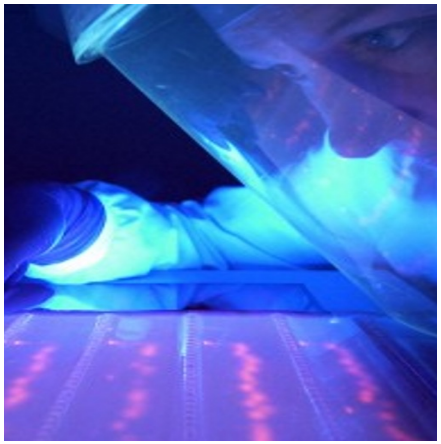
Methodology

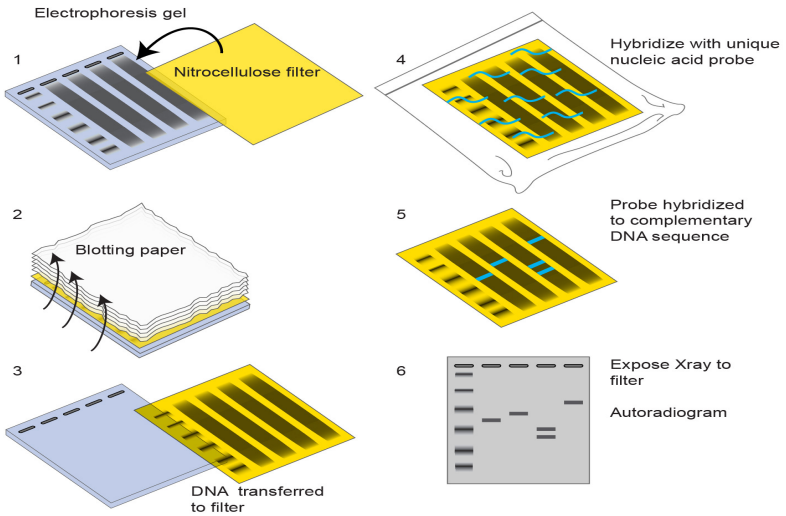
After transformation and selection step ,

- PCR analysis was used to examine the expressing of cas9 .
- Also Southern blot also used to examine the copy number of Cas9 in plant



https://www.yourgenome.org/facts/what-is-pcr-polymerase-chain-reaction





https://www.genome.gov/genetics-glossary/Southern-Blot

Result and discussion

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Result and discussion

Sterilization methods

- In this method leaf was used because it was usually available and callus was used because it enhanced organs production.so, after surface sterilization step the leaf was sterilized without adding of strong chemicals

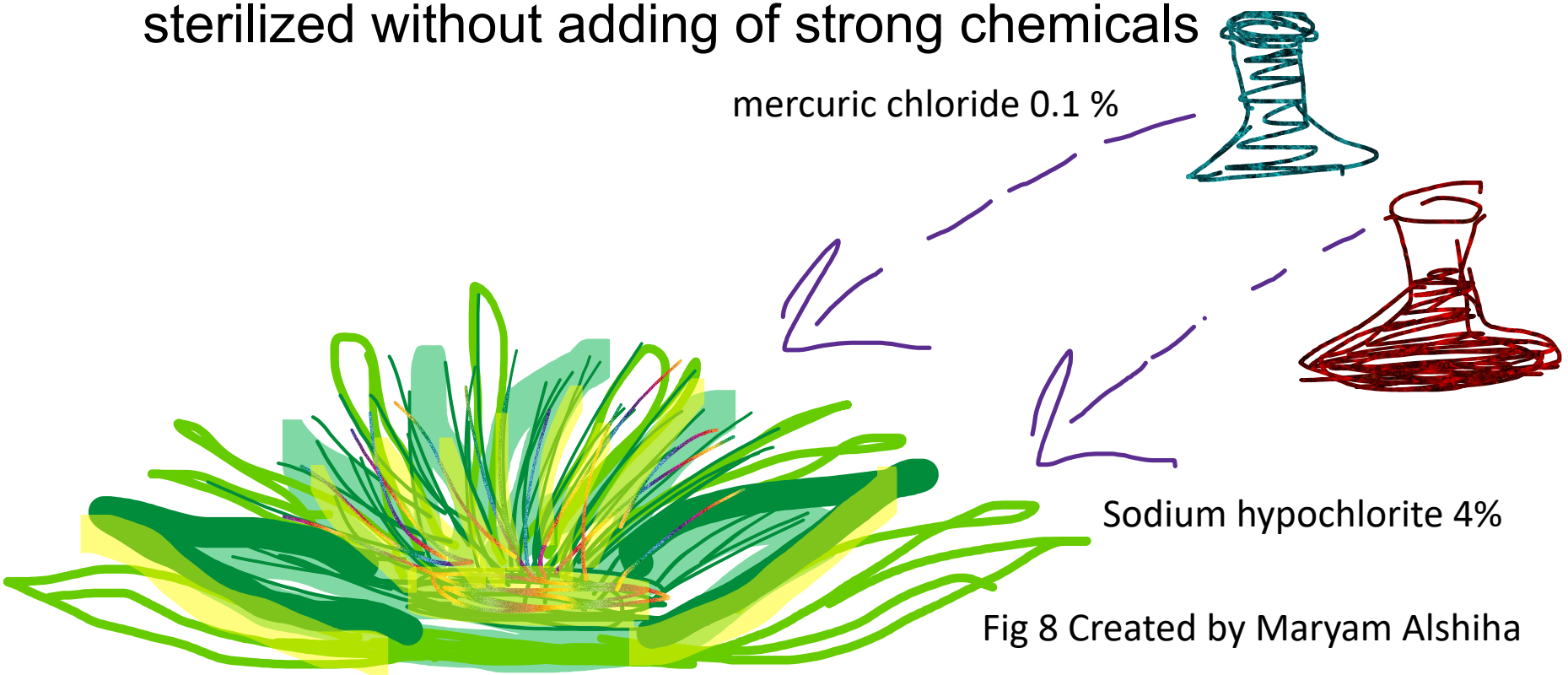


Fig 8 Created by Maryam Alshiha

Result and discussion

This table summarized the result of plant growth regulators effects

plant growth regulators effects corms **table 1**	**plant growth regulators effects somatic embryogenesis** **table 2**	**plant growth regulators effects cormlet formation from in vitro raised shoots** **table3**
When adding growth regulators, the yellow part began to appear two weeks after adding regulators as in Table 1, and the best results were shown by adding TDZ and NAA regulator, but it was noticed that callus is affected by temperature, so it was obtained under low temperatures. It was successfully obtained at 20C - 25C as table .	The TDZ and IAA PGR were enhanced and supported regeneration of cormles up to 70% . As result , TDZ with IAA were mixed and used that regulate and enhance plant growth and their biomass of somatic embryogenesis as table 2	Growth regulators were combined with sucrose by 6% because this ratio is the best for saffron renewal.as table 3

Result and discussion

- Table 1 show the result callus on MS media that was selected in different plant growth regulator . The best result was observed in TDZ and IAA that have beast biomass….

Table 1

Effect of plant growth regulators in combination with MS medium, on callus induction from corms of saffron

Combination of Plant growth regulators used					Response
BAP (mg/L)	NAA (mg/L)	2,4-D (mg/L)	TDZ (mg/L)	IAA (mg/L)	Number of explants forming calli/petriplate (n = 5) Mean ± SE
1.0	0.5	–	–	–	3.6 ± 0.00049
–	4.0	–	4.0	–	5.0 ± 0.0081
1.0	–	–	–	–	4.0 ± 0.0081
1.0	–	0.5	–	1.0	7.6 ± 0.0049
	–	1.0	–	1.0	3.8 ± 0.011
6.0	–	1.9	–	–	4.6 ± 0.049

Number of explants inoculated per petriplate was ten; five replicates of each combination were laid (total number of explants 50/combination). Data was collected after 8 weeks. Sucrose concentration in all the media was 3 g/L

(sudha chib,2020)

Result and discussion

- Table 2 show the result of somatic embryogenesis of saffron in different plant growth regulators

Table 2

Effect of plant growth regulators in combination with MS medium on somatic embryogenesis

Combinations of Plant growth regulators used							Response
BAP (mg/L)	NAA (mg/L)	TDZ (mg/L)	IAA (mg/L)	KIN (mg/L)	Picloram (mg/L)	Activated Charcoal (mg/L)	Number of somatic embryos formed/flask (n = 5) Mean ± SE
–	–	8.8	3.0	1.0	–	–	2.6 ± 0.0048
2.0	0.5	–	–	–	–	–	5.6 ± 0.0048
–	–	1.0	2.6	–	–	–	4.0 ± 0.008
–	–	0.5	–	–	0.5	–	4.4 ± 0.004
–	–	1.0	0.5	–	–	–	5.8 ± 0.012
–	–	0.5	1.0	–	–	0.1	8.4 ± 0.004

Number of explants inoculated per flask was ten; five replicates of each combination were laid (total number of explants 50/combination). Data was collected after 8 weeks. Sucrose concentration in all the media was 3 g/L

(sudha chib,2020)

Result and discussion

- Table 3 show the result of cormlet formation in vitro using MS (Murashing and Skoog media) with different plant growth regulators with sucrose to enhance the growth of saffron

Table 3

Effect of plant growth regulators in combination with MS medium on cormlet formation from in vitro raised shoots

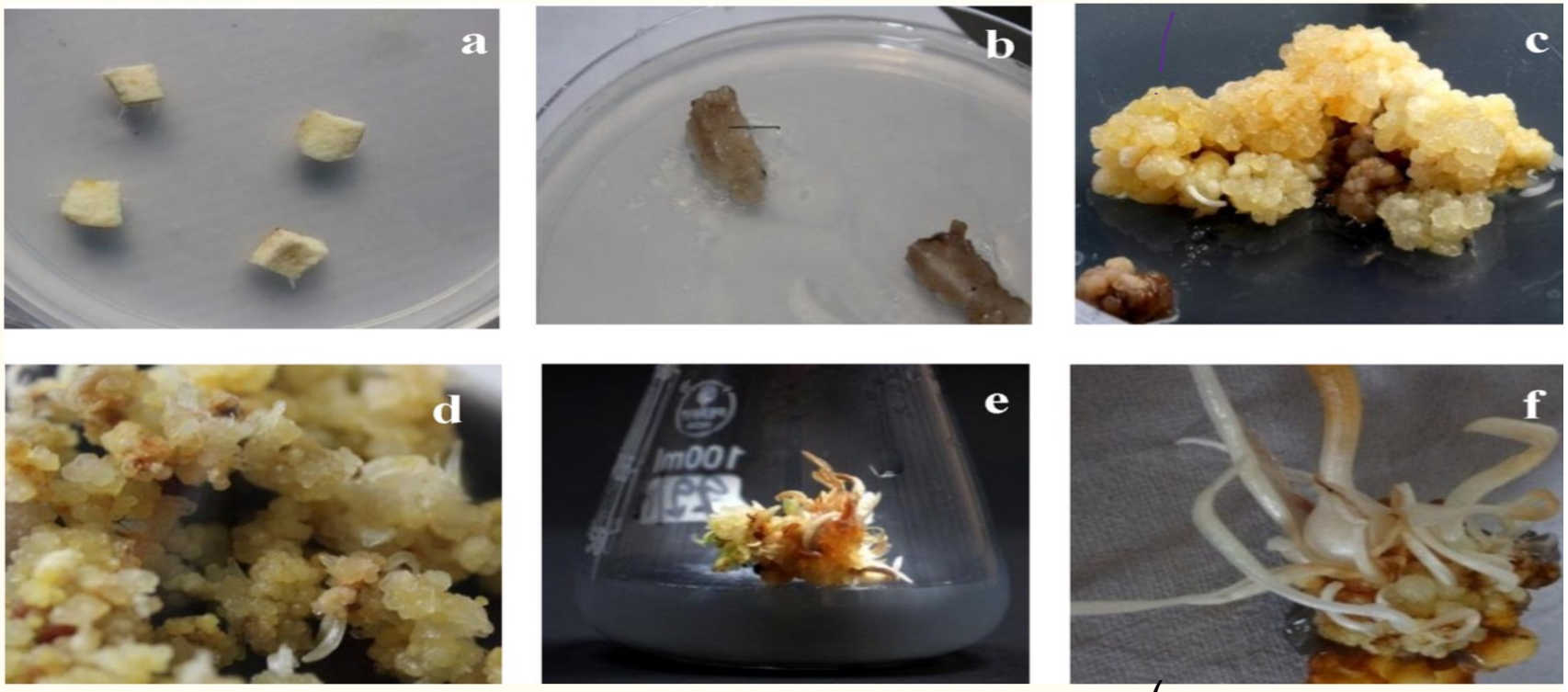
Combination of PGRs used with other supplements								Response
BAP (mg/L)	NAA (mg/L)	TDZ (mg/L)	IAA (mg/L)	IBA (mg/L)	Sucrose (g/L)	Activated charcoal (g/L)	Paclabutrazole (mg/L)	No. of cormlets formed/flask (n = 5) Mean ± SE
4.5	8.0	–	–	–	3.0	–	–	1.6 ± 0.0048
–	–	1.0	–	0.5	3.5	–	2.0	0.2 ± 0.0032
–	–	0.5	1.0	–	4.0	0.1	–	6.8 ± 0.004
–	–	3	2	–	3.0	–	–	2.0 ± 0.008
–	–	1	0.5	–	6.0	–	–	2.2 ± 0.004
–	–	1	0.5	–	8.0	–	–	1.2 ± 0.004

Number of explants inoculated per flask was ten; five replicates of each combination were laid (total number of explants 50/combination). Data was collected after 8 weeks

(sudha chib,2020)

Result and discussion

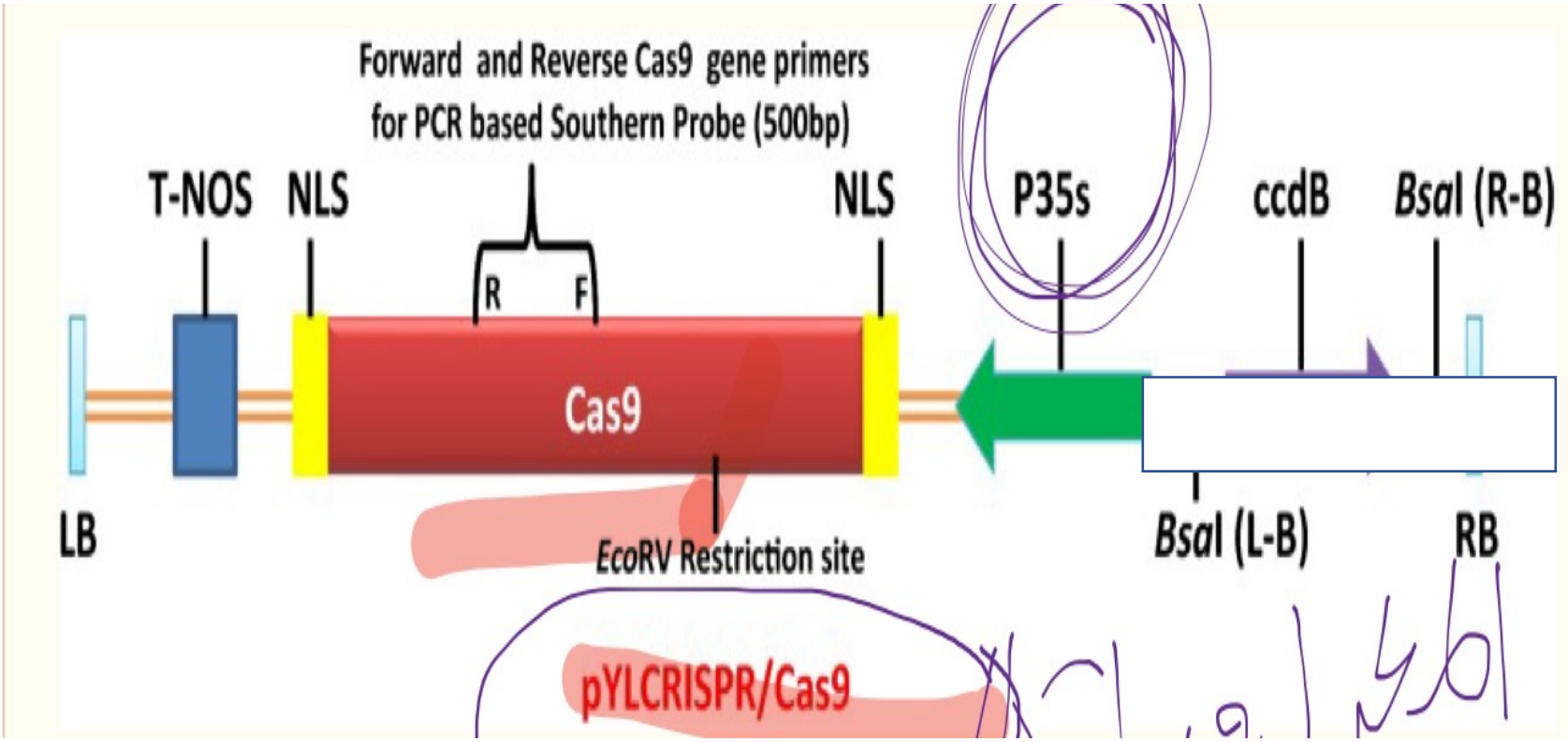
- Fig 9 show the development of cormlet and callus and generated under specifc condition with TDZ (plant regulater) that used…(a to f) to devoloped cormlets



(sudha chib,2020)

Result and discussion

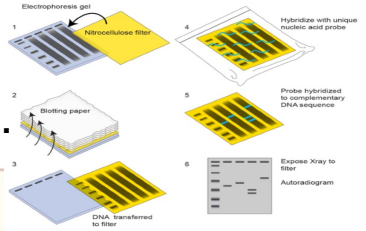
- Fig 10 show the successful integration of T-DNA in saffron genome using CRISPR/Cas9 tool

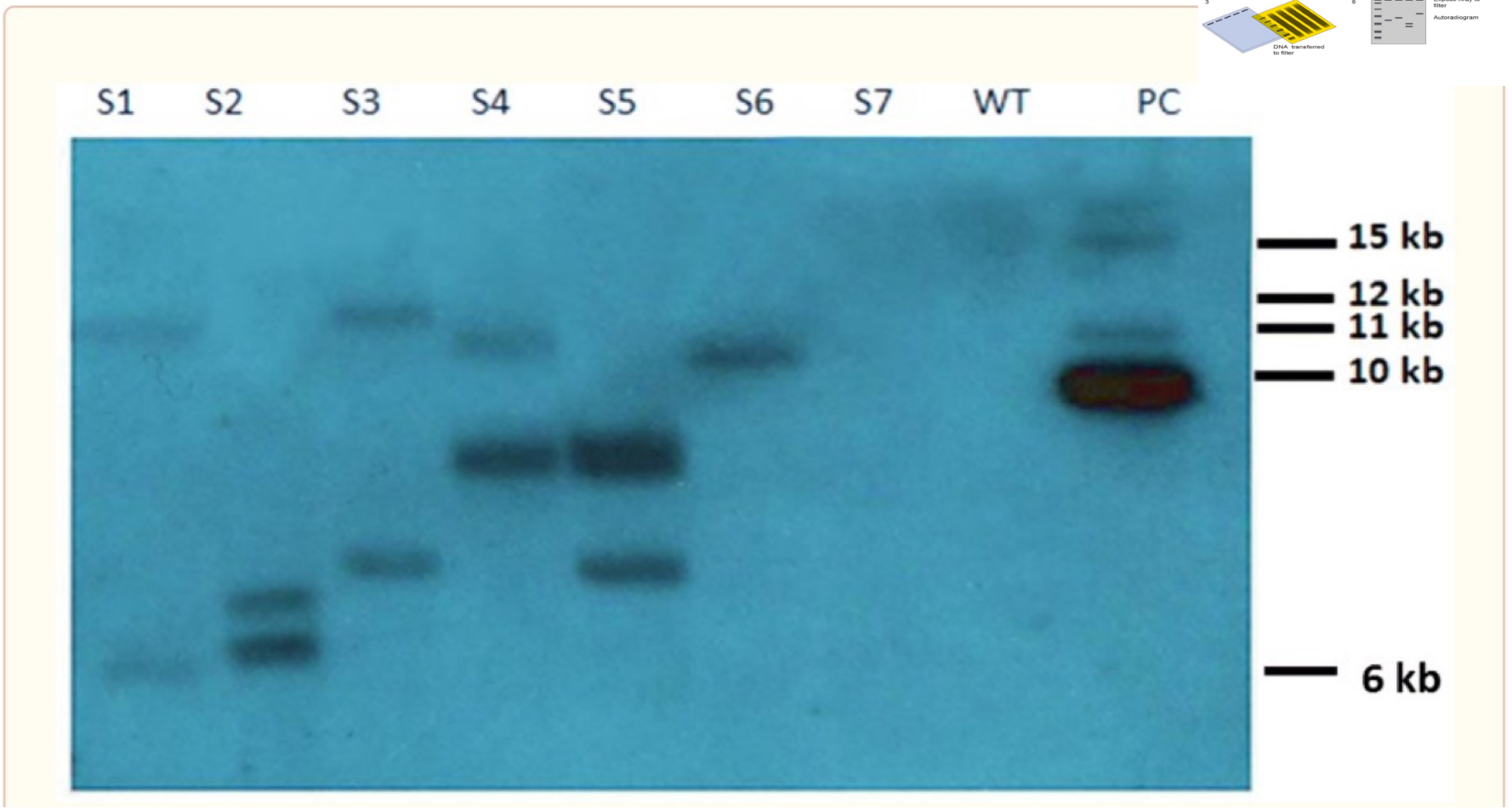


PYLCRISPR/Cas9p35s vector was derived from PCAMBIA1300 vector
(sudha chib,2020)

Result and discussion

- This fig 11 show the result of the number of Cas9 gene in different transgenic saffron plant .

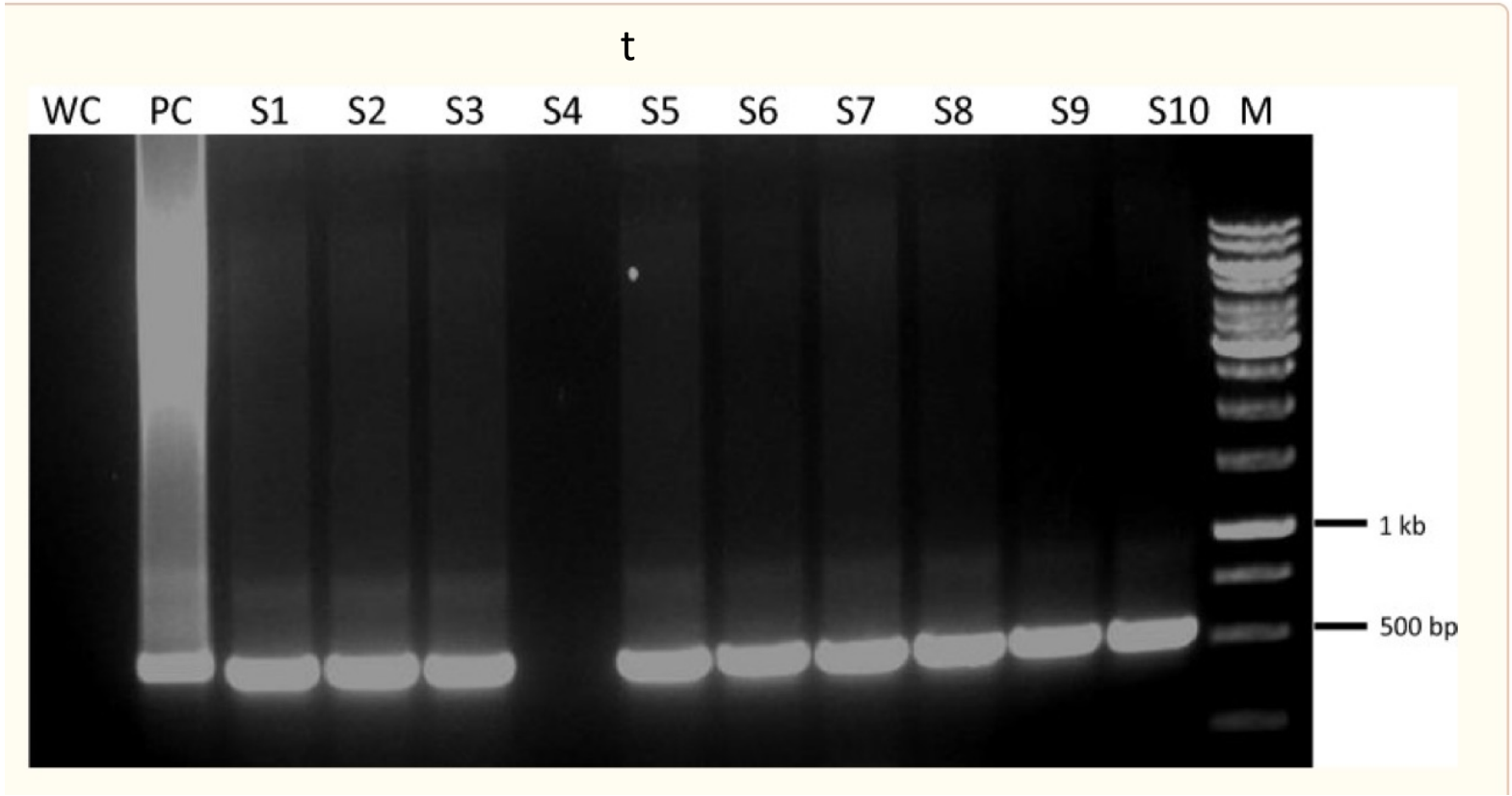




(sudha chib,2020)

Result and discussion

- Fig 12 show the band of cas9 gene expressing in Crocus sativus using PCR Analysis which appeared in all the gel except S4 which means that the transformation gene was successful except on S4



(sudha chib,2020)

Result and discussion

- **Tabel 4 shows the efficience transformation of Cas 9 in saffron .**
- **The highst transformation of efficiencies transgenic plant was detected was 4% in old somatic embryos culture with cefotaxime in 35 day**
- **Which means that the plant have successful interaction of Cas 9 gene into saffron and have ability to resiste bacteria .**

Table 4

Transformation efficiency relative to the experiment in which resistant callus or transgenic plants were produced

Type of explant	Age of the explant (in days)	Inoculated embryo (A)	No. of calli produced in media with cefotaxime	Transgenic plants (B)	Transformation efficiency (B/A %)
Immature embryo	30	52	2	0	0
Immature embryo	40	50	3	1	2
Immature embryo	35	50	4	0	0
Somatic embryo	30	120	8	3	3
Somatic embryo	35	56	5	2	4
Somatic embryo	40	98	6	3	3

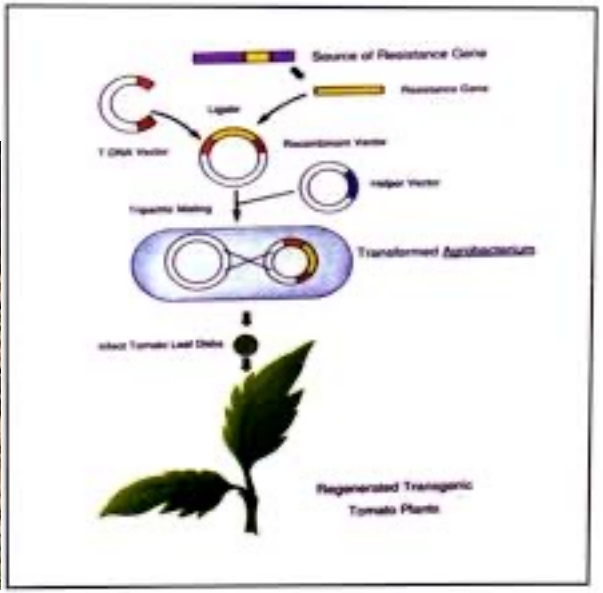
(sudha chib,2020)

Conclusion

- The selected paper
- Background about saffron production
- Paper objective
- Methodology
- Result and discussion
- Conclusion
- Possible application of saffron production in Kuwait
- Reference

Conclusion

- The developed biotechnology techniques have contributed to solving the problem of saffron production through genetic modification in its genome and editing its genes using CRISPR-Cas9 technology. Effective callus production has also been developed in plant production, which helps farmers in producing saffron and overcoming production difficulties.



Possible application of saffron production in Kuwait

- The selected paper
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Possible application of saffron production in Kuwait

- It is possible to apply this technique in Kuwait by applying it in hydroponic farms and trying to grow saffron without soil (aquatic tissue culture), which makes saffron resistant to climatic conditions.

Reference

- The selected paper
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Reference

- Chib S, Thangaraj A, Kaul S, Dhar MK, Kaul T. Development of a system for efficient callus production, somatic embryogenesis and gene editing using CRISPR/Cas9 in Saffron (*Crocus sativus* L.). Plant Methods. 2020 Apr 7;16:47. doi: 10.1186/s13007-020-00589-2. PMID: 32280363; PMCID: PMC7137501.

Thank you for Listening!

- You can enjoy drinking saffron tea with cake and ask me questions…