T.C.
ÖMER HALİSDEMİR UNIVERSITY
GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
DEPARTMENT OF AGRICULTURAL GENETIC ENGINEERING

OPTIMIZATION OF *AGROBACTERIUM TUMEFACIENS*-MEDIATED TRANSFORMATION IN ONION (*Allium cepa* L.)

KHAZINA AMIN

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Master Thesis

Supervisor

Assistant Professor Dr. Ali Fuat GOKCE

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Khazina AMIN tarafından **Yrd. Doç. Dr. Ali Fuat GÖKÇE** danışmanlığında hazırlanan **"Optimization of Agrobacterium Tumefaciens-Mediated Transformation in Onion (*Allium cepa* L.)"** adlı bu çalışma jürimiz tarafından Ömer Halisdemir Üniversitesi Fen Bilimleri Enstitüsü **Tarımsal Genetik Mühendisliği** Ana Bilim Dalı'nda Yüksek Lisans (İngilizce) tezi olarak kabul edilmiştir.

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THESIS CERTIFICATION

I certify that the thesis has been written by me and that, to the best of my knowledge and belief. All information presented as part of this thesis is scientific and in accordance with the academic rules. Any help I have received in preparing the thesis, and all sources used, have been acknowledged in the thesis.

KHAZINA AMIN

SUMMARY

OPTIMIZATION OF *AGROBACTERIUM TUMEFACIENS*-MEDIATED TRANSFORMATION IN ONION (*Allium cepa* L.)

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Ömer Halisdemir University

Graduate School of Natural and Applied Sciences

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This systematic study was undertaken to optimize the various factors affecting *Agrobacterium tumefaciens*-mediated transformation in onion, such as response of different onion cultivars, type of explants and composition of different plant growth regulators in callus inducing and regeneration media. *Agrobacterium* strain LBA4404 harboring binary vectorpBIN19 was used to infect explants. The pBIN19 vector contained *uidA* gene (interrupted by an intron) to identify putative transgenic shoots at earlier stage; it also contained *nptII* gene that encodes resistance against kanamycin. The results exhibited the genotype and explant dependency in onion transformation. Out of 355 primary transformants, 87 plants were recorded as positive when subjected to PCR assays; 51 belonging to cultivar Kral showing the tendency of genotype to genetic improvement. Molecular analysis (PCR) demonstrated that highest frequency of transgenic plants was contributed by mature embryos followed by seeds and basal plates. Onion seed as explant has been used for first time as no evidence in literature was found. The optimization of these factors will provide a gateway to introduce any desired trait in onion.

Keywords: Agrobacterium tumefaciens, onion, genetic transformation, regeneration, GUS analysis, transgenes.

ÖZET

SOĞANLARDA (*Allium cepa* L.) *AGROBACTERIUM TUMEFACIENS* İLE TRANSFORMASYONUN OPTİMİZASYONU

AMIN, Khazina
Ömer Halisdemir Üniversitesi
Fen Bilimleri Enstitüsü
Tarımsal Genetik Mühendisliği Bölümü

Danışman : Yrd. Doç. Dr. Ali Fuat GÖKÇE

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Bu çalışma, soğanda*Agrobacterium tumefaciens*aracılığıyla gerçekleştirilen transformasyona etki eden, farklı soğan çeşitleri, eksplant tipi, kallus indükleyici ve rejenerasyon ortamlarında kullanılan farklı bitki büyüme düzenleyicisi gibi faktörlerin optimize etmek amacıyla yürütülmüştür. LBA4404 *Agrobacterium* ırkı ve pBIN19 ikili vektörü bitkileri enfekte etmekte kullanılmıştır. pBIN19 vektörü, aday transgenik sürgünleri erken aşamada teşhis etmek için kullanılan *uidA*(intronla bölünmüş) geni ve kamaisine dirençlilği sağlayan *nptII* genini içermektedir. Yapılan çalışma sonunda, soğan transformasyonunun genotip ve eksplan tipine bağlı olduğun görülmüştür. 355 birincil transformantta yapılan PZR analizleri sonucu 87 bitkinin pozitif olduğu gözlenmiştir. Bu 87 bitkinin 51 tanesi Kral çeşidine aittir ve bu çeşidin genetik olarak geliştirilebilme potansiyelinin daha yüksek olduğunu göstermektedir. Moleküler analizlerlere (PZR) göre, transgenik bitki oluşturma sıklığı sırasıyla olgun embriyolar, tohumlar ve soğan tabanıdır. Literatür taramasında soğan tohumlarının eksplant olarak kullanıldığına dair bir çalışmaya rastlanmamıştır. Bu faktörlerin optimizasyonu soğana arzulanan özelliklerin aktarılmasında yeni bir kapı açacaktır.

Anahtar kelimeler: Agrobacterium tumefaciens, soğan, genetik transformasyon, rejenerasyon, GUS analizi, transgenler.

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SYMBOLS AND ABBREVIATIONS

Symbols	**Descriptions**
µM	Micro molar
mM	Mill molar
g/l	Gram per liter
mg/l	Milligram per liter
ml	Milliliter
µl	Microliter
µmol m^{-2} s^{-1}	Unit of measuring Light (Micromoles per meter square per second)
W	Watt
%	Percent sign
°C	Degree Centigrade
NaH_2PO_4	Sodium Di-hydrogen Phosphate
ddH_2O	Double-distilled water
nptII	Neomycin Phosphotransferase
uidA	β-glucuronidase reporter gene
bp	Base Pair
pH	Potential of Hydrogen

Abbreviations	**Descriptions**
FAOSTAT	Food and Agriculture Organization Statistical Databases
KPK	Khyber PakhtunKhwa (Province of Pakistan)
GOB	Government of Balochistan
DNA	Deoxyribonucleic acid
Ti plasmid	Tumor inducing plasmid
GUS	βeta-glucuronidase
MS medium	Murashige and Skoog
B5 medium	Gamborg Medium
BDS medium	Gamborg's B5 medium modified by Dunstan and Short (1977)

2, 4-D	2,4-dichlorophenoxyacetic acid
NAA	1-Naphthaleneacetic acid
BAP	6-Benzylaminopurine
GA3	Gibberellic acid
LB	Luria-Bertani medium
NOS	Nopaline Synthase gene
CTAB	Cetyl trimethylammonium bromide
PCR	Polymerase Chain Reaction
PZR	Polimerik Zincir Reaksiyonu
X-Gluc	5-bromo-4-chloro-3-indolyl-beta-D-glucuronic acid
EDTA	Ethylene Diamine Triacetic Acid
RSM	Regeneration Selection Medium

CHAPTER I

INTRODUCTION

Onion is a monocotyledonous vegetable belonging to genus *Allium* and family *Alliaceae*. The cultivation of onion dates back to 3000 BC, originated from central Asia. *Allium* genus (x=8) has more than 500 species (Eady, 1995). *Allium cepa* L., the common bulb-type onion, is commercially most important member of this family (Pike, 1986). Several edible species, closely related to *A. cepa*, include *A. ascalonicum* L. (shallot), *A. sativum* L. (garlic), *A. fistulosum* L. (Welsh onion or Japanese bunching onion), *A. ampeloprasum* L. (Leek, Kurrat and great-headed garlic), *A. tuberosum* L. (Chinese chives), *A. schoenoprasum* L. (chives) and *A. chinenese* Maxim (rakkyo). *Allium cepa* L. is either biannual or perennial depending on the region and cultivation conditions. Almost all of the species of *Allium* are native to the northern hemisphere of world and first domesticated in the mountainous areas of Pakistan, Afghanistan, Tajikistan and Iran (Brewster, 1994).

Onion is an indispensable item of human's diet due to its nutritional value and flavoring qualities. There is no limit to its use in diet as an essential condiment and vegetable by any nationality (Pike, 1986).

The cultivated onions are grown and eaten in many regions of the world. The tangy onions seem to receive much acclaim worldwide as it is the second most cultivated vegetable in the world with a total production of 84 million tons in the year of 2014 (FAOSTAT, 2014). China is leading onion producer with 20.8 million tonnes followed by India 8 million tonnes, United States of America 3.4 million tonnes, Egypt 2.2 million tonnes and Iran 1.92 million tonnes (FAOSTAT, 2014). Turkey ranks sixth among top onion producing countries in the world. The production of onion in Turkey was reported 1.9 million tonnes in the year of 2014 (FAOSTAT, 2014). Pakistan stands seventh largest onion producing country in the world with the total production of 1.76 million tonnes in the year of 2014 (FAOSTAT, 2014). Onions produced by Indo-Pak region are famous for their best quality, pungency and availability round the year. The major onion growing areas in Pakistan are, Awaran, Kalat, Chagi, Mastung, Turbat and Khuzdar in Balochistan, Mirpurkhas, Hyderabad, Sukkar,

Sanghar, Badin and Naushero Feroze in Sindh, Swat district in KPK and Gujranwala, Kasur, Vehari, Jhang, Sheikhupura, Khaniwal and D.G. Khan in Punjab (GOB, 2014).

From the ancient times, the conventional plant breeding has been a great tool to improve the crops from economical point of view. The problem with the conventional plant breeding methodologies is their unpredictability as it may lead to several years of careful greenhouse work of breeding to develop a plant with desirable characteristics (DANIDA, 2002). Genetic transformation technologies have enabled the scientists to introduce any trait of economic importance in crops by breaking the barriers across species. These technologies transcend traditional plant breeding methodologies by allowing the rapid and predictable gene transfer across the species boundaries (Pimental et al., 1989). Potential benefits include herbicide resistance, insecticide resistance and enhanced nutritional profile of crops (Pimental et al., 1989).

Agrobacterium tumefaciens-mediated transformation is one of the most reliable methods of genetic transformation in plants. It is being used for genetic transformation of many crops. The protocol of *Agrobacterium* meditated transformation is relatively simple, straightforward, economical, and the most important; it results in the insertion of single transgene (Hansen and Wright, 1999). This transformation system has been long established in dicotyledonous plants (Fraley et al., 1986). Many desired traits have been introduced in plants by using transformation and regeneration methods. However, most monocotyledonous plant species are reluctant to *Agrobacterium*-mediated transformation. It led to the development of direct gene transfer techniques in monocots species. But these direct gene transfer techniques did not praise much by scientists mainly due to insertion of multiple copies of transgene (Christou, 1995; Christou, 1997).

Onion belongs to monocotyledonous group and it's a difficult crop for genetic transformation. The optimization of the factors influencing *Agrobacterium tumefaciens* mediated transformation efficiency will provide a gateway to introduce any desired trait in onion. On the frame of an improved strategy directed to strengthen the genetic transformation system in onion by using *Agrobacterium tumefaciens*, any desired gene can be introduced in plant in order to make them agronomically superior (Lydiate et al., 1995).

Development of an efficient and reproducible plant transformation and regeneration protocol is a prerequisite for genetic transformation studies of plants (Sivanesan et al. 2015). Successful plant transformation requires a proper DNA delivery system, a plant regeneration system, and a selection system to recognize the transgenic cells. For onion, the characterization of these transformation aspects is still lacking and needs to be optimized for an efficient genetic transformation (Ramakrishnan et al. 2013).

Keeping in view the economic importance of onion with desired traits, the current research work has been done in order to optimize genetic transformation system in onion. This study has explored the various factors affecting *Agrobacterium tumefaciens* mediated transformation in onion. Factors affecting transformation efficiency, such as response of different onion cultivars, type of explants and effect of different plant growth regulators on onion regeneration have been studied. The expression of the *uidA* gene coding for βeta-glucuronidase is used as an indicator in the optimization of transformation protocol. Overall transformation efficiency in onion elite lines has been checked. The results documented herein may be helpful for the future research regarding introduction of any desired gene in onion by using this optimized transformation strategy.

CHAPTER II

LITERATURE REVIEW

2.1 Early Attempts of Genetic Transformation in Plants

Genetic transformation has been a worthwhile goal for scientists to improve the crops of economic value. It allows direct manipulation of a plant genome for desired characteristics. Fraley et al. (1983) was the first who developed a genetically modified tobacco plant conferring antibiotic resistance. The Ti region of *Agrobacterium tumefaciens* plasmid fused with an antibiotic resistant locus which resulted into a chimeric gene. The tobacco plants were infected with *Agrobacterium tumefaciens* having this chimeric region followed by selection of transformed tobacco cells through tissue culture techniques.

Farley et al. (1986) documented that *Agrobacterium* mediated transformation system had been well established in plants belonging to dicotyledonous species. A broad host range of *Agrobacterium* to form tumors had been detected in many dicotyledonous plants. In monocotyledonous plants, *Agrobacterium* rarely had been observed to form gall crown tumors. Initial attempts to transform the monocots plants were unsuccessful. Scientists believed that monocotyledonous plants were comparatively recalcitrant to *Agrobacterium* mediated transformation. Pitzschke et al. (2010) stated that *Agrobacterium* species are broad-host range plant pathogens, which causes tumor in most dicotyledonous and some monocotyledonous plant species. The *Agrobacterium*-mediated transformation in plants involves multiple steps and the concerted action of both bacterial and host factors (Figure 2.1).

Graves and Goldman (1986) conducted transformation experiments on maize seedlings in which assaulting bacterial strains of *Agrobacterium* showed tendency to infect and transfer of T-DNA in to host maize plant. These transformation results in monocots encouraged other scientists to test different strains of *Agrobacterium* and a variety of monocot plant tissues. Hiei et al. (1994) reported an efficient *Agrobacterium* transformation protocol in rice after studying the response of different *Agrobacterium* strains to a variety of tissues. Screening of

transformed cells was done by GUS testing and a hygromycin resistance gene was used as a selectable marker. The results gave high transformation frequencies in rice.

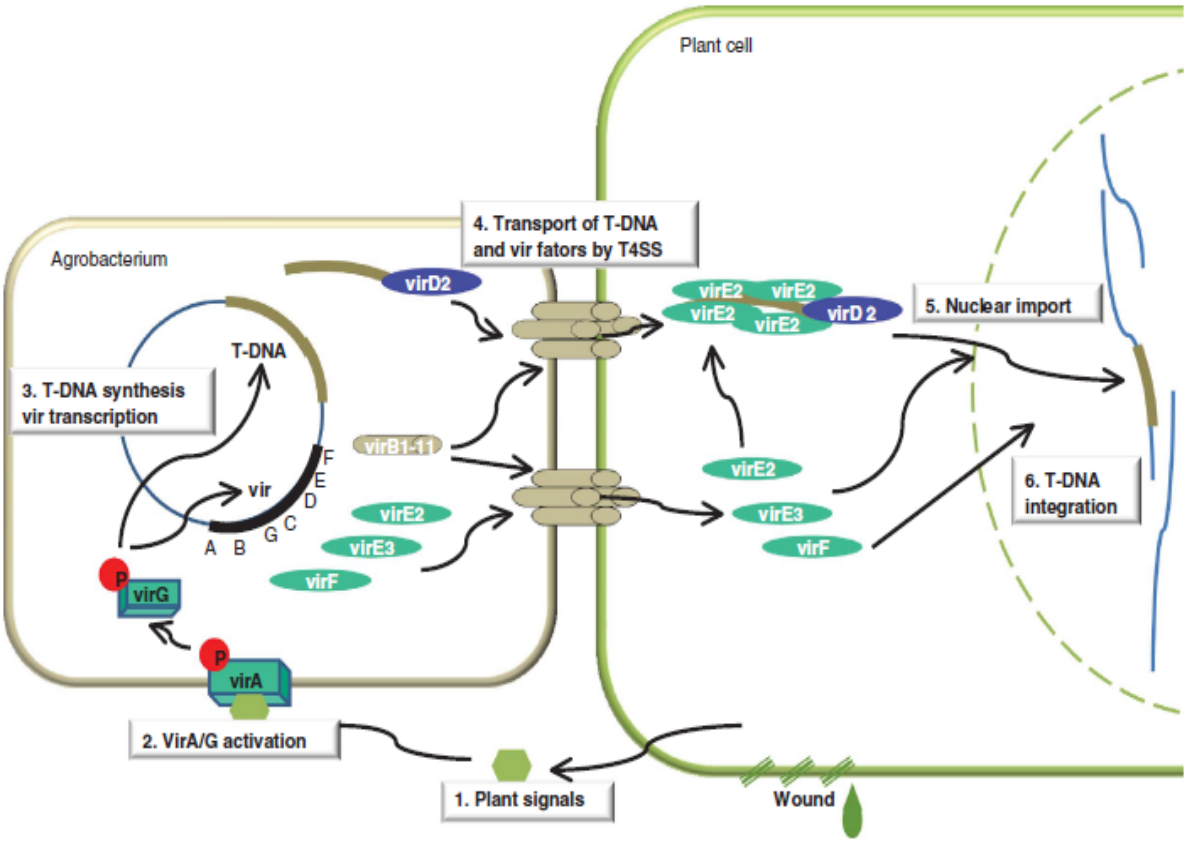


Figure 2.1. Overview of the *Agrobacterium*–plant interaction**.** 1. Induction of plant signals, 2. Activation of Vir A/G,3. Synthesis of T-DNA followed by vir gene expression in *Agrobacterium*, 4. Through a bacterial type IV secretion system (T4SS) T-DNA and Vir proteins are transferred into the plant cell to assemble a T-DNA/Vir protein complex, 5. The T-DNA complex is imported into the host cell nucleus, 6. The T-DNA becomes integrated into targeted host plant chromosomes by illegitimate recombination (Pitzschke et al., 2010).

Klein et al., (1987) and Christou et al., (1992) used particle bombardment method for plant transformation. Aragão et al., (2000) reported that particle bombardment mediated transformation could be proved more convenient method to achieve transformation in crop plants as compared to *Agrobacterium* mediated transformation without any host range limitations. However, Altpeter et al., (2005) documented that particle bombardment method may led to successful integration of two or more transgenes in host cells, in addition to the selectable marker and the maximum copy number of transgenes reported to date was 13. Vaucheret et al., (1998) and Dai et al., (2001) reported that gene silencing was the result of integration of more copy number or large fragments of transgenes.

Several different methods for direct DNA transfer in plants were also reported in literature including microinjection (Crossway et al., 1986), transformation of protoplasts mediated by chemicals like calcium phosphate or polyethylene glycol (Datta et al., 1990), electroporation (Fromm et al., 1986), embryogenic suspension culture method (Finer and McMullen, 1991) and plant transformation by using silicon carbide whiskers (Frame et al., 1994). Pimental et al., (1989) stated that genetic transformation technologies allow the rapid and predictable gene transfer in crops across the species boundaries. Potential benefits such as herbicide resistance, pest resistance, better flavor and enhanced nutritional profile of crops make these technologies more efficient.

2.2 Genetic Transformation System in Onion

Arumuganathan and Earle (1991) reported that initial attempts of genetic transformation in onion were likely to prove this crop recalcitrant. Onion being monocotyledonous crop poses less response to transform genetically. It has large genome size which may reduce the likelihood of transgene integration at active site of genome and probability to have correct transgene expression may affect. Due to these reasons, onions may prove a difficult crop to transform genetically. An optimized and functionally stable protocol having a reasonable number of transformants may solve this problem.

Elomaa et al. (1993) and Robinson and Froozabady (1993) also stated that the plants with high ploidy levels or extremely large genome sizes would be more challenging to transform

as compared to related diploid plant species having small genomes. The larger plant genome may reduce the probability of integration of transgene at functionally active region. Even after transformation, the foreign DNA often fails to express efficiently. Eady et al. (1995a) reported that failure of transgene expression may be the result of DNA condensation. Torres et al. (1993a) considered that methylation may be the reason behind failure of foreign gene integration and stable expression in plant species. This phenomenon may be avoided by using methylation-minus *Agrobacterium* strains to deliver DNA in targeted plant so the transformation stability may be achieved.

Kamstaityteet al. (2004) studied the main factors which limited the efficiency of in-vitro onion regeneration. They reported that bulblet formation, tissue virtification and plantlets dormancy were the main reasons to decrease the efficiency of onion micropropagation. Dommisse et al. (1990) reported that onion, like other monocotyledonous crop species, showed its tendency to be a host to *Agrobacterium tumefaciens.* Eady et al. (2000) first ever reported genetic transformation in onion. He gave successful *Agrobacterium* transformation protocol for immature zygotic embryos. But this approach had a drawback of unavailability of explant round the year.

Zheng et al. (2001) carried out transformation experiments in onions and shallots. He reported an efficient and reliable *Agrobacterium* mediated transformation strategy by using three week old onion calli induced from mature zygotic embryos. Zheng et al. (1998, 1999) stated that seeds would be more advantageous and feasible to use for onion transformation due to easy storage and availability round the year.

Eady et al. (2000) and Zheng et al. (2001) published initiative reports of successful *Agrobacterium* mediated transformation in onion and shallot. Kondo et al. (2000) performed transformation experiments in garlic. In these initial transformation protocols, embryos were used as the explants. Hansen and Wright (1999) and Gelvin (2003) independently reported that *Agrobacterium* mediated transformation would be more efficient and cost effective method. In the experiments, they performed or studied, this method resulted in the insertion of single or fewer copies of transgene, resulted in less gene expression problems.

There were several other methods which had been used to transform recalcitrant plant species. In all these methods, the DNA or transgene delivery systems were different. Datta et al. (1990) reported chemical methods (calcium phosphate or polyethylene glycol) for genetic transformation of recalcitrant crop plants. Fennel and Hauptman (1992); Xu and Li (1994) used electroporation to transform monocotyledonous crops. Kaeppler et al. (1992); Thompson et al. (1994) used silicon fibers as transform delivery system for crop species reluctant to genetic transformation. Crossway et al., (1986) conducted experiments of genetic transformation in monocots by using microinjection.

Christou et al. (1991); Somers et al. (1992); Weeks et al. (1993) and Wan and Lemaux (1994) independently used particle bombardment method for genetic transformation of different monocotyledonous crops. They conducted transformation experiments and showed that this technique worked well for such recalcitrant plant species. Kaeppler et al. (1992) and Thompson et al. (1994) introduced a new technique to transform monocotyledonous crop plants called silicon fiber mediated transformation. They used silicon fibers to puncture and deliver transgene into targeted cells. The results were similar to the biolistic particle gun method. This technique was easier to handle as compared to gene gun method thus posed less cell damage during transformation. Delbreil et al. (1993); Hiei et al. (1994) stated that development of different transgene delivery systems for recalcitrant plant species would be necessary because of insensitivity of plants to bacterial infection.

2.3 Genotype Effects in Onion Transformation

Valk et al. (1992) investigated that an efficient transformation and regeneration system of onion did not depend on genotype. But different genotypes responded differently on regeneration medium. There were large differences in their response to regeneration system. Many onion cultivars used in regeneration studies were highly heterozygous. Sometimes, variation within those onion cultivars was higher than variations among cultivars. Phillips and Hubstenberger (1987) reported that the hybrids of *Allium fistulosum* x *Allium cepa* were highly responsive to regeneration system. Eady et al. (1995b) documented that “Yellow Express” also known as “Sapporo Yellow” onion cultivars gave relatively best response to in-vitro experiments.

Regeneration from protoplasts is very difficult task and highly dependent to genotype. Buitveld and Creemers-Molenaar (1994) firstly regenerated leek plants by using suspension cultures. Those suspension cultures were derived from certain leek embryos. The frequency of those leek embryos was very low. Buitveld and Creemers-Molenaar (1994) investigated the effect of different leek genotypes on regeneration cultures and they concluded that regeneration from protoplasts was highly dependent to genotype and frequency of having successful transformation was extremely low.

Eady et al. (2003) recovered herbicide tolerant onion plants from immature onion embryos. They documented that transformation procedure was independent to onion cultivar used. The maximum onion transformation efficiency was 0.9 %. Hailekidan et al. (2013) developed an efficient in-vitro regeneration protocol for shallots. They documented that the transformation and regeneration frequency in shallots was highly influenced by genotype.

2.4 Different Explants Used in Onion Transformation

Zheng et al. (1998); Zheng et al. (2001) and Zheng et al. (2005) documented various successful reports on onion transformation and regeneration using mature embryos as explants. Eady et al. (1998a); Eady et al.(1998b); Eady et al. (2000) and Eady et al. (2003) used immature embryos for onion transformation. Eady (1995) reported that different onion explants were used for in-vitro regeneration system to obtain calli. He enlisted them in his review study along with their degree of regeneration. Basal plates, inflorescence, shoot tips, roots, leaf base, twin scales, embryos, aerial bulbs, ovules and anthers had been tested and used for onion in-vitro regeneration since 1981.

Viterbo et al. (1992) reported that the most efficient callus systems were derived from basal plates and embryos. The calli derived from basal plates were able to regenerate de novo. The cells of basal plates were totipotent and highly competent to accept foreign DNA as they contained the source of progenitor cells.

Rabinowitch and Brewster (1990) documented that field grown onion bulbs were not good explants. There was some doubt in their callus totipotency. The amount of explant after surface sterilization was very less as their surface sterilization was a difficult task itself.

Furthermore, origin of regeneration was unclear from results whether regeneration arose from single cells of onion bulbs or was the result of rearrangement of some dedifferentiated cells into an organized and differentiated structure. Rabinowitch and Brewster (1990) revealed that most onion calli usually showed less response to regeneration. A high proportion of onion cells within a callus were important to obtain good frequency of transformants.

Buitveld et al. (1994); Valk et al. (1992) and Shahin and Kaneko (1986) conducted experiments using onion embryos or seedlings derived calli. The callus derived from embryo showed high regeneration frequency. These embryogenic cultures were similar to those used in the genetic transformation of other monocotyledonous crop plants. Vasil et al. (1992) stated that onion embryos would be an efficient and suitable explant for genetic transformation. The embryos must be dissected from presoaked seeds. This would be a tedious and time consuming procedure.

Eady et al. (1995b) used onion seedlings as explant in genetic transformation studies. They derived seedlings from microbulbs which formed after germination of seeds on media germination. They supplemented medium with picolarm to obtain microbulbs. These microbulbs contained dedifferentiated cell mass which produced multiple shoots. Newly emerged shoots were sub-cultured to shooting medium. Microbulbs were transformed by using two strategies; particle bombardment method and *Agrobacterium* mediated transformation. Gus assay results revealed that this culture system gave stable integration of transgene in both transformation methods tested.

Hussey and Falavigna (1980) conducted experiments by using twin scales on onion as in-vitro culture material. They reported that onion twin scales provided a good source of dedifferentiated cell mass. The cluster of dedifferentiated cells at the base of scales showed adventitious regeneration. Due to compactness of these cells, as they were situated at the base of scales, delivery of foreign DNA was difficult.

Dommisse (1993) used onion split stems as explants. He derived split stems from secondary shoots of twin scales. He cultured them on medium supplemented with different hormones. Regeneration via organogenesis or embryogenesis was observed. He reported that split stems

would prove a good in-vitro culture material for transient assay studies. The inoculation process of onion split stems with *Agrobacterium* was similar to that used for tobacco leaves.
Marinangeli et al. (2005) investigated effect of different onion explants on callus induction. They tested different explants of onion "Valcatorce INTA" including; mature zygotic embryos, immature umbel, fecundated ovules, basal plates and apical meristems with basal plates. All explants derived calli showed less regeneration potential having an average of 6.6 %. However, immature embryos showed high regeneration potential (73.1 %). The calli derived from zygotic embryos were highly variable in regeneration potential (6.5-42.8 %).

Marinangeli et al. (2005) focused that type and source of explants were important factors plant regeneration in onion. They also tested different levels of hormones concentrations to check their effects on callus induction and revealed callus induction was more dependent on type of explants as compared to auxin type or its concentration. The calli of zygotic embryos and ovules, induced by 2, 4-D, were more crumbly and showed less root differentiation as compared to those induced by picloram. Ashwath et al. (2006) conducted transformation experiments using callus derived from seedling radicles. They concluded that seedling radicles were relatively superior to other explants due to their easy manipulation and high transformation rate.

Xu et al. (2007) developed particle bombardment mediated transformation method for onions. They used stem discs as explants to obtain embryogenic calli. The experiment was designed to introduce a zinc-finger protein gene "OSISAP1" into onion to raise the salinity and alkali tolerance. The source of this stress associated protein gene was *Oryza sativa* subspecies *indica*. Kamata et al. (2011) revealed that onion callus obtained after pre-conditioning of suspension culture callus on solid medium performed well in *Agrobacterium* mediated transformation. They were more amenable to transformation process. The calli was easily observed and distinguished under microscope with or without yellowish auto-fluorescence.
Ramakrishnan et al. (2013) developed an efficient system of somatic embryogenesis in onion. They used onion shoot apex as explants. The frequency of primary callus induced on MS medium supplemented with 2, 4-D was 85.3 %.

Malla et al. (2015) studied micro-propagation and DNA delivery system in onion. In their study, they obtained maintainable onion calli using twin scale leaves and basal plates. They tested effect of different concentrations of acetosyringone on callus induction. In-vitro onion bulb response was observed on MS medium supplemented with hormones. They gave a successful protocol which ensured onion regeneration by using basal plates and twin scale leaves as explants. Wu et al. (2015) studied somatic embryogenesis in onion. They modified onion regeneration protocol in order to get high frequency of in-vitro plantlet regeneration. They selected mature embryos as explant for regeneration experiments.

2.5 Effect of Media and Plant Growth Regulators

The onion culture requirements of media and plant growth regulator for clonal propagation are not definite. To achieve proliferation, MS, B5 or BDS based media in combination with usual cytokinins and auxins have been used. Eady (1995) stated that different authors used different explant source, genotypes, experimental conditions and methods of measuring results. Therefore, comparison between different treatments usually may not be possible. Nitsch and Nitsch (1969) used complex vitamin mixtures for plant regeneration. Phillips and Luteyn (1983) documented that specific requirements were necessary for the regeneration of onion plants from callus. They improved callus induction by using picloram in medium.

Shahin and Kaneko (1986) used BDSx media which contained BDS macronutrients and MS micronutrients supplemented with casein hydrolysate (900mg/l). Marani et al. (1994) conducted regeneration experiments in garlic. They evaluated that use of cytokinin thidiazuron significantly increased the efficiency of proliferation of garlic microshoots from meristem tip cultures. Kamstaityteet al. (2004) studied onion micropropagation. They used MS medium supplemented with NAA, BAP and kinetin. They investigated the effect of BAP and kinetin in different concentration. The outcomes revealed that high concentration of growth regulators increased the number of microshoots. When the BAP concentration in medium was increased from 0.9 to 4.4 µM, there was a significant increase in microshoots per explant. However, high concentration of BAP had negative effect on plant regeneration. The highest regeneration frequency was obtained by using 10.6µM kinetin in regeneration

medium that was 1.9 to 2.1 microshoots per explant. The regeneration ability was enhanced when BAP used in comparison with kinetin.

Ramakrishnan et al. (2013) developed an efficient system of somatic embryogenesis in onion. Callus was derived from shoot apex on MS medium supplemented with 2, 4-D (4.0 mg/l). The frequency of primary callus induction on MS medium supplemented with 2, 4-D was 85.3 %. After sub-culturing, them on MS medium supplemented with 2, 4-D (2.0 mg/l), embryogenic callus was obtained. The addition of a low concentration of BAP, glycine (2.0 mg/l), proline (690 mg/l) and casein hydrolysate (1.0 g/l) enhanced the frequency of embryogenic callus induction. The regenerated plantlets from calli were shifted to half strength MS medium supplemented with IBA (1.5 mg/l) to enhance root development.

Malla et al. (2015) studied micro-propagation and DNA delivery system in onion. The results of this study revealed that maximum callus induction was observed on MS medium fortified with B5 vitamins in addition with picloram (0.5-1 mg/l). The frequency of maximum plant regeneration from callus was observed on MS medium supplemented with BAP and Kinetin (0.5 mg/l each) and NAA (0.1 mg/l). To obtain in-vitro onion bulbs, they used MS medium fortified with B5 vitamins in addition with BAP (2.0 mg/l).

Wu et al. (2015) studied somatic embryogenesis in onion. They modified onion regeneration protocol in order to get high frequency of in-vitro plantlet regeneration. They selected mature embryos as explant in regeneration experiments. The effect of different ionic concentration, thiamine (Vitamin B_1) and sucrose in MS medium was investigated. The highest callus induction frequency was obtained when ionic concentration of NH_4^+ / NO_3^- was set to 30/30 mM /mM. Somatic embryo maturation was favored on 20 g/l sucrose and 10 mg/l V_{B1} in MS medium. The highest rate of somatic embryo germination was recorded 93.3 %.

2.6 Selection System for Onion Transformation

Wilmink and Dons (1993) documented that the plants which showed the ability to grow in the presence of any herbicide or antibiotic were selected as they were transgenic plants. Different plants tissues responded differently to selective agents on medium. Mostly,

aminoglycoside antibiotics were used by scientists for the selection of transgenic plants. Alternatives of antibiotics, herbicides are being used as selective agents. Herbicide "Basta" hinders the activity of enzyme glutamine synthetase due to the presence of L-Phosphinothricin (PPT) as an active ingredient. "Bar" gene (encodes enzyme Phosphinothricin-Nacetyl Transferase) is conferred resistant against this agent. This enzyme uses acetyl coenzyme-A as a cofactor and inactivates PPT by acetylation. Wang et al. (1992) and Wilmink and Dons (1993) demonstrated that onions were more sensitive to herbicide "Basta" as compared to antibiotics kanamycin, G418 or hygromycin.

Vasilet al. (1993) documented that herbicide "Basta" was proved helpful in recuperating transgenic plants from tissues which were chimeric for the "Bar" gene. Downs et al. (1994) used phosphinothricin as a selective agent in media. It supplemented deficient cells and allowed improved growth. Kramer et al. (1993) reported that selection efficiency was increased by adding chlorophenol red indicator to the phosphinthricin containing media. Sensitive cells grown on this amass ammonia caused medium to change color.

Dhir et al. (1994) studied the effect of anthocyanin marker gene system. This system simplified the selection of transgenic plants due to presence of anthocyanin pigment in the transformed cells. This system allowed visual indication of transgenic materials at any stage of the regeneration process rather than using antibiotics or herbicides.

Aswath et al. (2006) proposed a novel selection system for onion transformation that did not need any herbicides or antibiotics to select transgenic plants on medium. In onion selection system, they used *manA* gene which encoded for phosphomannose isomerase (*pmi*). The source of (*pmi*) gene was bacteria (Escherichia coli). Transgenic plants carrying the *manA* gene that coded for *pmi*. The embryogenic calli originated from seedling radicles were used for transformation procedure. The transformation rates calculated for *Agrobacterium*-mediated transformation and biolistic gene gun method were 27 % and 23 % respectively. Xu et al. (2007) developed particle bombardment mediated transformation method for onions to introduce a zinc-finger protein gene "OSISAP1" into onion to raise the salinity and alkali tolerance. A binary vector harboring *nptII* gene coffering kanamycin resistance and GUS

reporter gene was used to identify and select transgenic plants. GUS reporter system gave visual identification of transformed callus due to GUS activity.

2.7 Targeted Traits to Improve Onion by Genetic Manipulation

Grant (1988) documented that the demand for red, mild sweet and early market onion has been increased. Onion flavor production is caused by secondary metabolite pathway. Particular flavor onions could be produced as "Flavr Savr" tomato is produced by successfully using the similar technology. These tomatoes have the ability to ripe naturally on the vine and have long shelf life; as a result, these are flavored tomatoes.

Dhir et al. (1994) studied that the genes responsible for anthocyanin, extracted from maize and inserted in onion, could be helpful in altering bulb color. Eady (1995) stated that several genes are controlling the earliness of onions as it is required specific day length and temperature due to which altering maturity rates in onions to meet early market would not be practicable at present.

In monocots, the bar resistance gene is most commonly used for the transformation and is considered as one of the first gene to be introduced in onions. Eady (1995) also revealed that herbicide resistant genes (bar and pat) could be introduced in onion lines to resolve the hybrid seed selection and post-emergence weed problem. Homozygous herbicide resistant line was crossed with herbicide sensitive line, as a result sensitive line hybrid (resistant) and selfed (sensitive) seeds were produced. By the application of proper herbicide at post emergence stage would led to the removal of selfed seed and weeds. Eady et al. (2003) recovered herbicide tolerant onion plants from immature onion embryos. The maximum transformation efficiency in their experiments was 0.9 %. Their study revealed that transformation procedure was independent to onion cultivar. The transformation strategy described could be used with different selective agents.

Jaynes et al. 1993 studied bacterial diseases such as soft rot caused by *Pseudomonas* or white rot caused by *Sclerotium cepivorum*. They stated that these bacterial diseases could be overwhelmed by the production of transgenic plants. By incorporating the genes coding for

antimicrobial peptides might reduce bacterial wilt in plants. Logemann et al. (1994) investigated that downy mildew (*Peronosporaceae*), a fungal disease, may demonstrate more difficulty to overwhelm. Chitinase and P-l,3-glucanase genes may increase resistance against fungal disease as showed against *Fusarium oxysporium* in tomato. Engelen et al. (1994) worked on "Plantibodies". They demonstrated that genes encoding artificial antibodies raised against specific pathogen antigens, when introduced into plants, conferred a high degree of protection. This approach may prove successful for onions.

Insect resistance genes may be introduced to control the major pest of onions like thrips (*Thrips tabacii*). Thomas et al. (1994) demonstrated that there was great reduction in whitefly (*Bemisia tabacii*) emergence (98.5 %) by the addition of tryptophan decarboxylase gene from *Cantharanthus roseus* into tobacco as compared to non-transgenic controls. These arrangements might be useful in onions against thrips. Zheng et al. (2005) produced insect resistance shallots having resistance against beet armyworm (*Spodoptera exigua*). They introduced a *H04* hybrid gene and *cry1Ca* gene to targeted shallot plants by using *Agrobacterium* mediated transformation method. The source of these genes was *Bacillus thuringiensis*. Transgenic shallots were obtained with an average transformation frequency of 3.68 %. The results suggested that *cry1Ca* and *H04* genes conferred resistance against beet armyworm in shallots.

Xu et al. (2007) developed particle bombardment mediated transformation method for onions. The experiment was designed to introduce a zinc-finger protein gene “OSISAP1” into onion to raise the salinity and alkali tolerance. The source of this stress associated protein gene was *Oryza sativa* subspecies *indica*. The results revealed clear difference in ability to tolerate stress. Non-transgenic plants were effectively killed by salinity of 200 mM/l, while transgenic plants were unaffected by salinity stress of 400 mM/l. Similarly, other genes coffering abiotic stresses in plants may be introduced in onion by using genetic transformation technologies.

An efficient and reproducible transformation system for onions has not yet been achieved. Transgenic plants can be produced in onion plants if plenty of time and energy is spent on developing regeneration, selection and delivery system as showed by recent advances and

success in recalcitrant plants. According to Eady (1995), it would take time to achieve an efficient and reproducible system of onion transformation and regeneration, as a few groups have been investigating. The problem does not lie with the delivery of the DNA or the competence of the cells to receive a foreign gene. The problem lies rather in the selection and particularly in the regeneration of stable functional transgenic onion tissue. Research in the problem areas in onion would hopefully improve the possibilities of transforming other commercially viable *Allium* species. To achieve these landmarks for onions may take several years due to the recalcitrant nature of onion crop for transformation and also little work is done related to onion transformation and regeneration aspects. The prospective benefits to the consumer such as particular flavor characteristics, choice of color, and improved storage have made onion transformation a worthwhile goal.

CHAPTER III

MATERIAL AND METHODS

3.1 Seed Sterilization

Onion seeds were surface sterilized by immersing in 100 ml sterile water containing two drops of Tween-20, for 15-20 minutes with continuous agitation, followed by 5-10 washings with sterile water in order to remove residues. After that, seeds were further surface disinfested by immersing in 70 % alcohol for 2 minutes and 2 % H_2O_2 for 15-20 min and then rinsed in sterile deionized water 4-5 times. The seeds were dried on autoclaved filter paper.

3.2 In-vitro Germination Test

Nine onion breeding lines were taken for initial in-vitro germination test to check the viability of seeds (Table 4.1). Sterilized seeds were grown on basal MS medium containing Murashige and Skoog (1962) mineral salts, 3 % sucrose and 7-8 g/l agar (pH= 5.6 - 5.8). 25 seeds in each magenta box were grown. Each variety was tested in 3 replications. After one week, in-vitro germination percentage for each breeding line was calculated as:

In-vitro Germination % = seeds germinated on MS medium /total seeds grown x 100

The cultivars with better germination % were selected for next step of genetic transformation.

3.3 Seed Culture Conditions

Sterilized seeds were cultured on basal MS medium containing 4.4 g/l mineral salts, 30 g/l sucrose and 7-8 g/l agar. The pH of medium was maintained 5.6 - 5.8. Cultures were grown under controlled conditions with a 16/8 hours light/dark photoperiod, 47 µmol m^{-2} s^{-1} irradiance and 58 W fluorescent tubes in the growth chambers. The temperature was set as 25 ± 2 °C. After one month, plants were ready to use for transformation procedure.

3.4 Preparation of Bacterial Culture

Agrobacterium strain harboring pBIN19 binary vector with *uidA* reporter gene was already available in plant transformation laboratory, Department of Agricultural Genetic Engineering, Omer Halisdemir University. The glycerol stock was streaked on LB plates containing kanamycin at concentration 50 mg/l and Rifampcin 100 mg/l. One colony from plate was inoculated in Luria-Bertani broth (LB broth) supplemented with appropriate concentrations of antibiotics. The bacterial culture was incubated in thermo-shaker at 28 ^{o}C for overnight.

3.5 Preparation of Explants

Next day, mature embryos were obtained from presoaked seeds. Sterilized seeds were soaked on filter paper in the presence of sterile deionized water to make filter paper wet. Mature embryos started to emerge from seed coat after 36-48 hours. By using of 2 forceps, gently took out mature embryos. Root tips, shoot tips, leaf blades and basal plates were excised from in-vitro grown plants and inoculated.

3.6 Inoculation and Co-cultivation with *Agrobacterium* Suspension

Onion cultivars were subjected to *Agrobacterium* mediated genetic transformation using different explants (Shoot tips, leaf blades, root tips, basal plates, seed and mature embryos). LBA4404 strain harboring BIN19 binary vector containing *uidA* gene (βeta-glucuronidase) under the control of 35S promoter was used (Figure 3.1). It is noteworthy here that *uidA* gene was interrupted by an intronic sequence so that the expression may result only from eukaryotic cells. The explants were inoculated with *Agrobacterium* suspension (O.D 0.6) for 40 minutes with gentle shaking in liquid medium without antibiotic followed by incubation on co-cultivation medium for three days approximately. Co-cultivation medium contained MS salts including vitamins, 3 % sucrose, 7-8 g/l agar and 100 µM acetosyringone. The pH of medium was adjusted to 5.6 - 5.8. Co-cultivation was carried out in growth chamber using standard culture conditions.

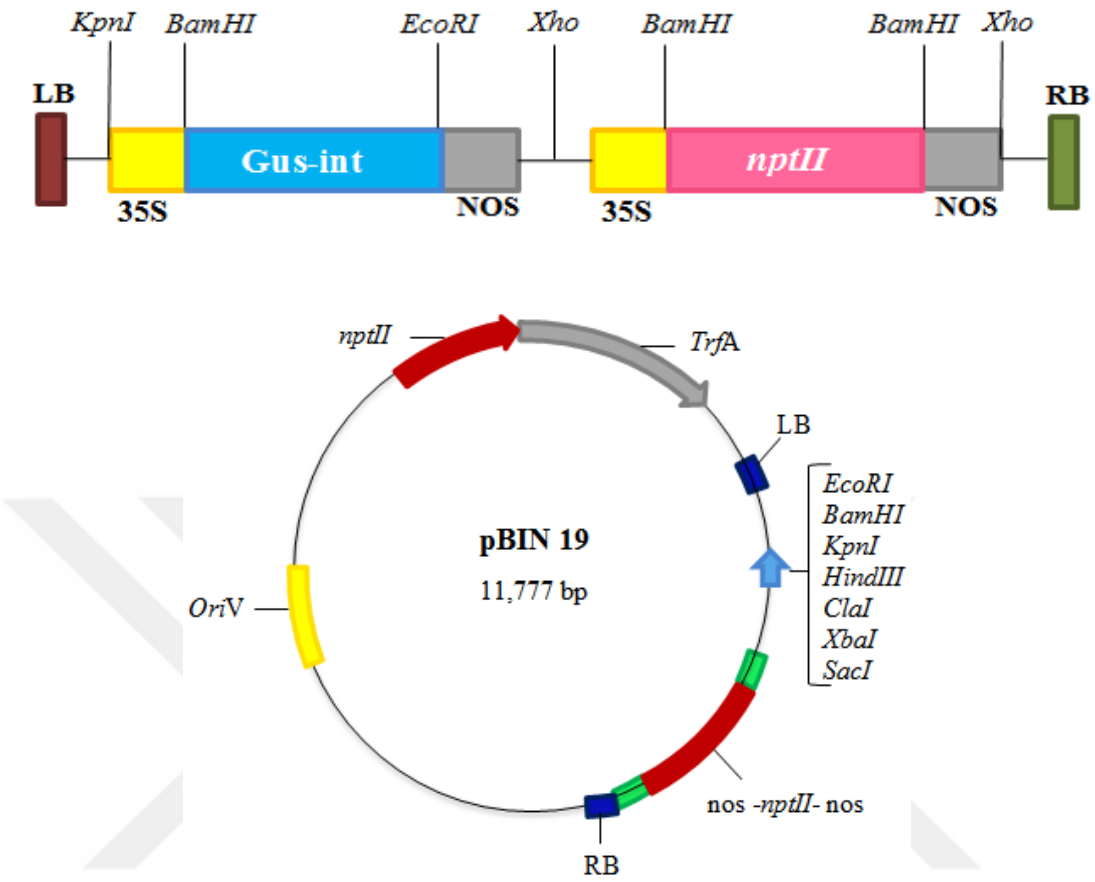


Figure 3.1. Schematic representation pBin19 containing βeta-glucuronidase (*uidA*) and neomycin phosphotransferase (*nptII*) driven by cauliflower mosaic virus (CaMV35S) promoter and nopaline synthase (NOS) terminator in between right and left border. The construct has *nptII* gene that encodes resistance to Kanamycin, which was used as a plant selectable marker at 100 mg/l concentration.

3.7 Regeneration Selection Media

Following co-cultivation with *Agrobacterium*, the explants were washed well in antibiotic solution (Broad spectrum antibiotic Duocid with ingredients of ampicillin + Sulbactam Sodium) to wash away the bacteria on the surface of explants. After five minutes, took them out and dried on filter paper followed by culturing on regeneration selection medium (RSM). Three different RSM were tested to optimize the conditions for in-vitro onion regeneration (Table 3.1).

Table 3.1. Composition of Different RSM used in Onion in-vitro Regeneration

Reagents	RSM-1	RSM-2	RSM-3
MS salts	4.4 g/l	4.4 g/l	4.4 g/l
Sucrose	30g/l	30g/l	30g/l
pH	5.6-5.8	5.6-5.8	5.6-5.8
Agar	7-8 g/l	7-8 g/l	7-8 g/l
BAP	2ml/l or (2mg/l)	0.05 ml/l or (0.05 mg/l)	2ml/l or (2mg/l)
NAA	0.2 ml/l or (0.2 mg/l)	--	2ml/l or (2 mg/l)
2,4-D	--	0.5 ml/l or (0.5 mg/l)	--
Kanamycin	2ml/l (100 mg/l)	2ml/l (100 mg/l)	2ml/l (100 mg/l)
Duocid	5ml/l (500 mg/l)	5ml/l (500 mg/l)	5ml/l (500 mg/l)

RSM-1 included MS medium supplemented with 2 mg/l BAP, 0.2 mg/l NAA, 500 mg/l Duocid (to suppress the growth of *Agrobacterium*) and 100 mg/l Kanamycin (for plant selection). RSM-2 included MS medium supplemented with 0.05 mg/l BAP, 0.5 mg/l 2,4-D, 500 mg/l Duocid and 100 mg/lKanamycin.RSM-3 included MS medium supplemented with 2 mg/l BAP, 2 mg/l NAA, 500 mg/l Duocid and 100 mg/l Kanamycin. BAP and NAA were used in equal concentrations. BAP and NAA were added before autoclave, while kanamycin and duocid were added after autoclave just before to pour medium in glass petri plates.

3.8 Transfer to Shoot/Root Medium

With well-developed calli and micro-shoots were transferred to shoot/root medium (MS medium supplemented with 2 mg/l BAP, 0.2 mg/l NAA, 1 mg/l GA3, 500 mg/l Duocid and 100 mg/l Kanamycin).

3.9 Acclimatization

When plantlets reached to a certain size in shoot elongation media with rooting as well, putative transgenic plants were shifted to pots containing 3:1 mixture of organic matter and perlite. They were transferred to soil and shifted in green house.

3.10 GUS Histochemical Assay

The expression of *uidA* gene was studied through histochemical X-Gluc assay. GUS solution was prepared containing 10 mg/L X-Gluc, 10 mM EDTA, 100 mM NaH_2PO_4, 0.1 % Triton X-100 and 50 % methanol. The pH was adjusted to 8.0. The solution was protected from light. The regenerated shoots/explants transformed were dipped in X-Gluc solution in an eppendorf and kept at 37 ^{0}C for one hour to overnight.

3.11 Molecular Evaluation of Putative Transgenic Plants

Total 355 primary transformants were recovered from in vitro regeneration experiments. They were subjected to PCR analysis to confirm the presence of introduced gene. DNA extraction was done by CTAB method according to the protocol given by Murray et al., (1980). DNA concentrations were checked through Nanodrop spectrophotometer. DNA dilutions (5 ng/µl) were made through formula:

$$\mathbf{C_1V_1 \ : C_2V_2}$$

Where,

$\mathbf{C_1}$= known concentration (ng/µl)

$\mathbf{V_1}$ = known volume of DNA solution

$\mathbf{C_2}$= Required concentration (ng/µl)

$\mathbf{V_2}$ = Required volume

PCR analysis was done to confirm presence of transgene in plants. For 1x PCR reagents, 12.5 µl 2x-mix, 0.5 µl (10 mM) of each forward and reverse primer, 5 µl (5 ng/µl) DNA dilution and 6.5 µl ddH2O were mixed to make 25 µl total reaction volume. PCR program for *nptII gene* was set for 35 cycles (initial denaturation at 95 ^{o}C for 5 minutes, denaturation at 94 ^{o}C for 1 minute, annealing at 58 ^{o}C for 45 seconds, extension at 72 ^{o}C for 1 minute, final extension at 72 ^{o}C for 7 minutes and hold at 4 ^{o}C for infinite time. PCR program for *uidA gene* was set for 35 cycles (initial denaturation at 95 ^{o}C for 5 minutes, denaturation at 94 ^{o}C for 30 seconds, annealing at 55 ^{o}C for 30 seconds, extension at 72 ^{o}C for 1 minute, final extension at 72 ^{o}C for 7 minutes and hold at 4 ^{o}C for infinite time.

For gel electrophoresis, 1.5 % agarose gel and 0.5x TBE buffer were used. 50 bp DNA ladder was used to check the band size of desired gene. The plasmid DNAs were used as positive controls in both PCRs (for *nptII* and *uidA* genes). For wild control, non-transformed DNA "B17-50B" (provided by Dr. Gökçe, A.F.) was used.

3.12 Calculation of Transformation Efficiency

After compiling up all data, the effect of genotype and hormones on transformation efficiency was evaluated. To determine Mean Transformation Efficiency (MTE) of each targeted explant for *Agrobacterium* mediated transformation we counted number of transgenic plants regenerated (nTPR) in total number of explants used (tnEU) and used those values in the formula;

$$MTE\ (\%) = (nTPR / tnEU) \times 100$$

CHAPTER IV

RESULTS

4.1 In-vitro Germination of Seeds

Nine cultivars of onion were used to compare in vitro germination among cultivars. The germination percentage for each variety was calculated. Two onion cultivars "Sampiyon" and "Kral", with average germination percentages of 93 % and 78 % respectively, were selected for the proposed study. These two cultivars were selected on the basis of good in-vitro response and seed germination percentages.

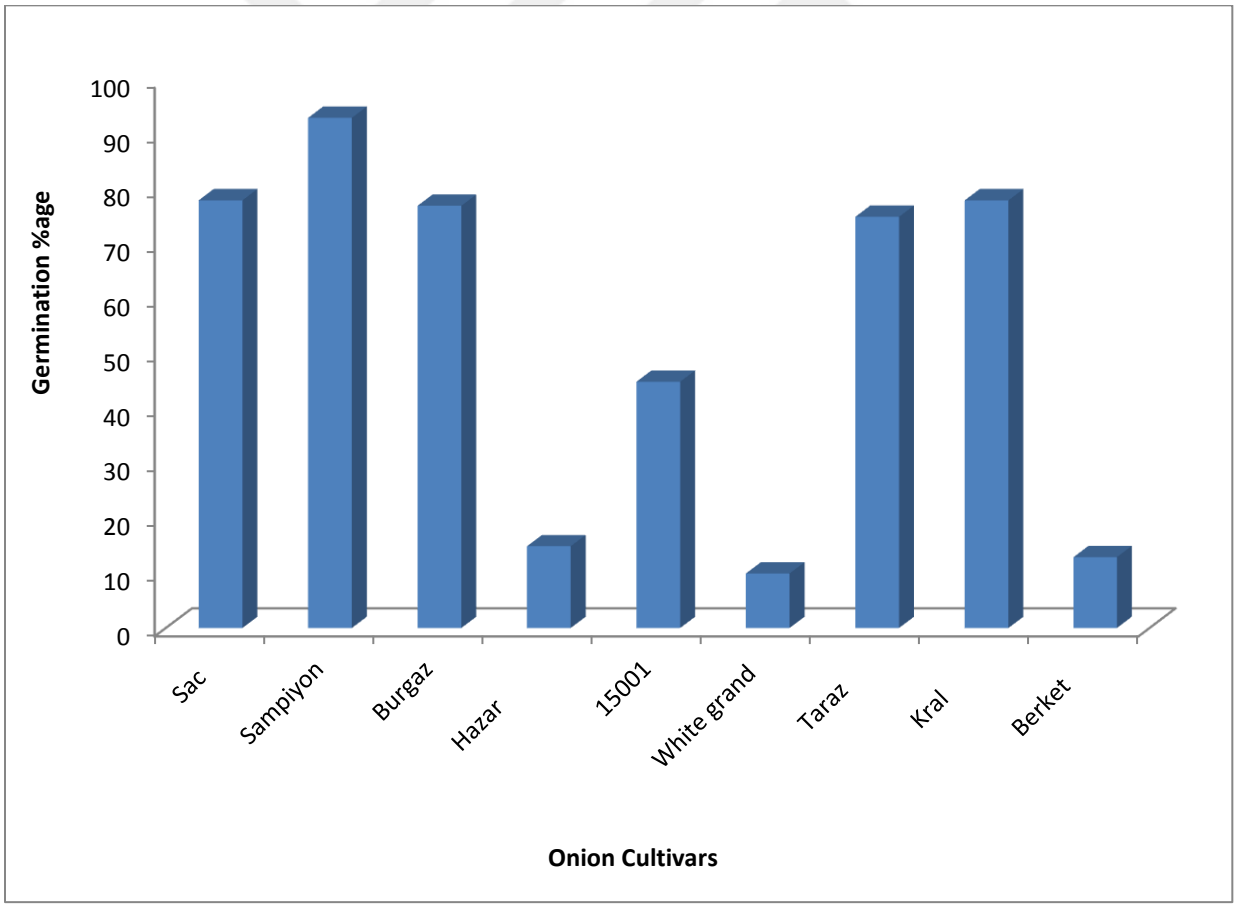


Figure 4.1. In-vitro germination % of seeds of different onion cultivars.

4.2 Seed Culture and Preparation of Mature Embryos

Seeds were sterilized prior to use in each in-vitro experiment to avoid any contamination in cultures. Sterilized seeds were grown on MS basal medium to get explants (shoot tips, root tips, leaf blades and basal plates). Mature embryos were obtained from presoaked sterilized seeds.

Figure 4.2. Seed Sterilization. (a) seeds immersed in sterile water containing Tween-20, (b) washing of seed with sterile water to remove residues.

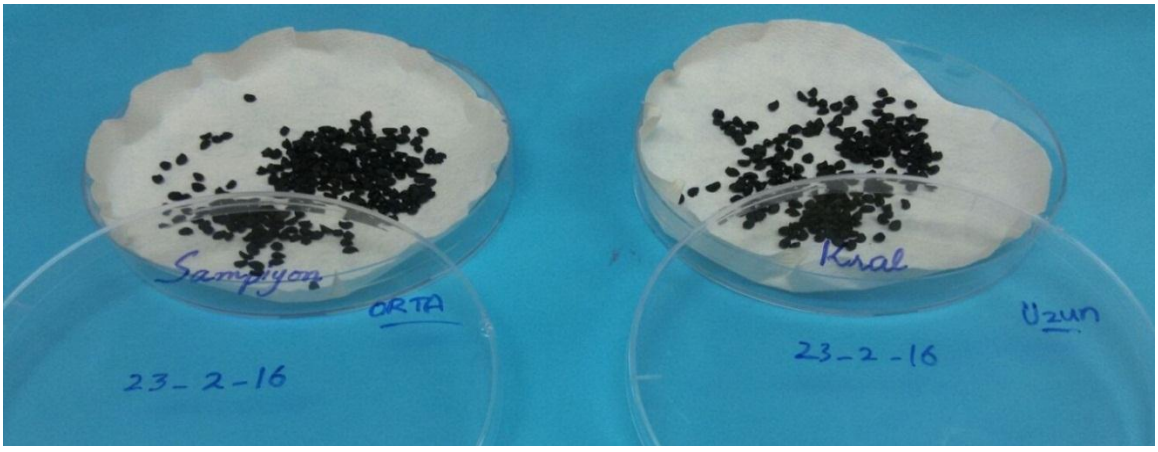


Figure 4.3. Drying of seeds on sterile filter paper after sterilization.

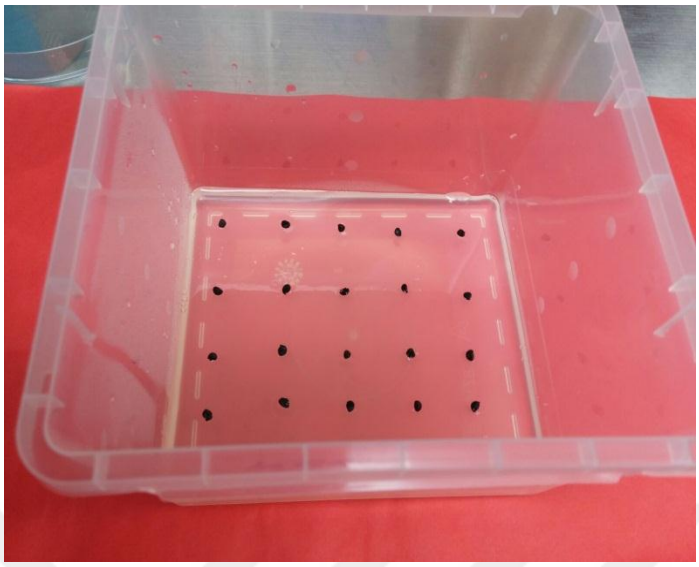


Figure 4.4. Sterilized seeds grown in magenta box on MS basal medium.

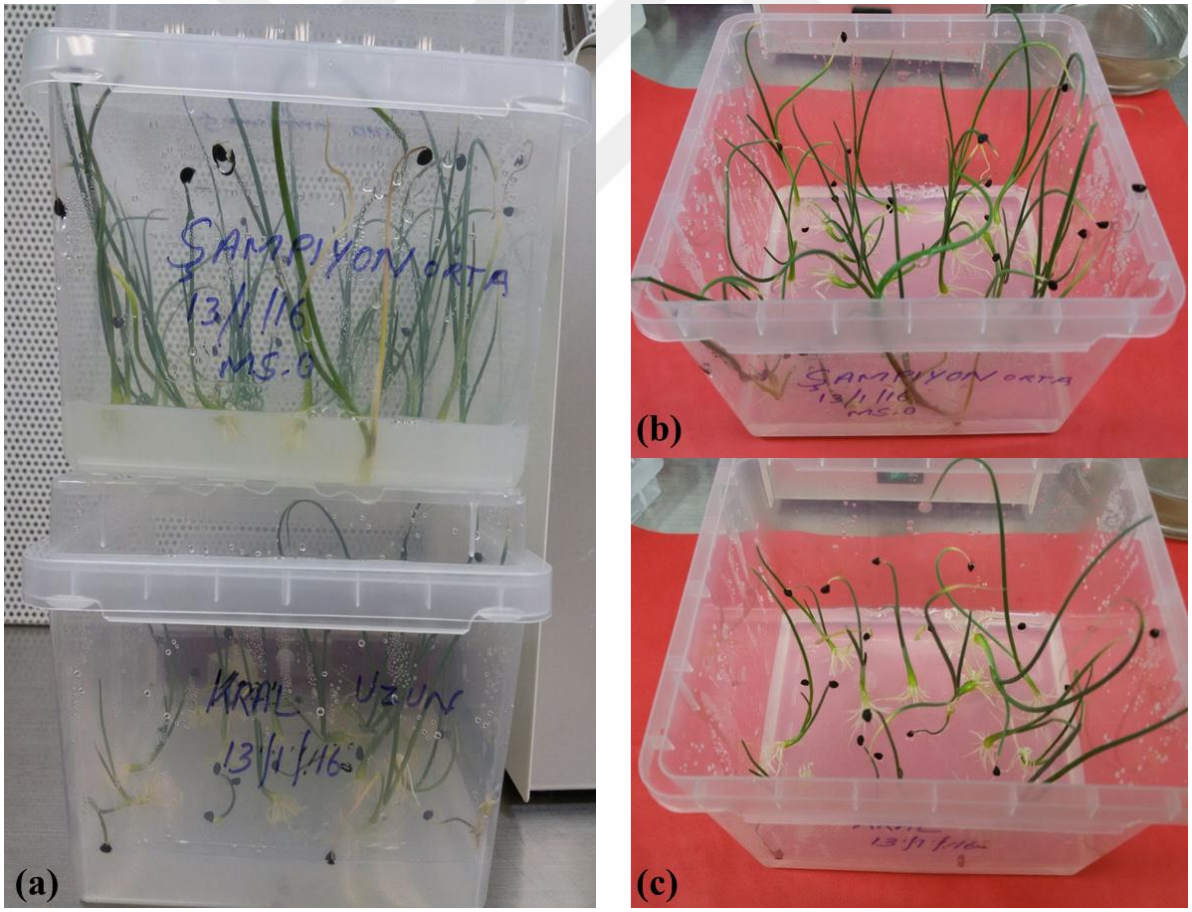


Figure 4.5. (a) In-vitro onion plants obtained after one month of growing on MS basal medium, (b) Sampiyon plants, (c) Kral plants.

Figure 4.6. (a) Pre-soaked onion seeds on wet sterile filter paper, (b) white colored onion mature embryos ready to use as explant.

4.3 Inoculation and Co-Cultivation with *Agrobacterium* Suspension

Different onion explants (Shoot tips, leaf blades, root tips, basal plates, seed and mature embryos) were subjected to inoculation with *Agrobacterium* suspension for 40 minutes.

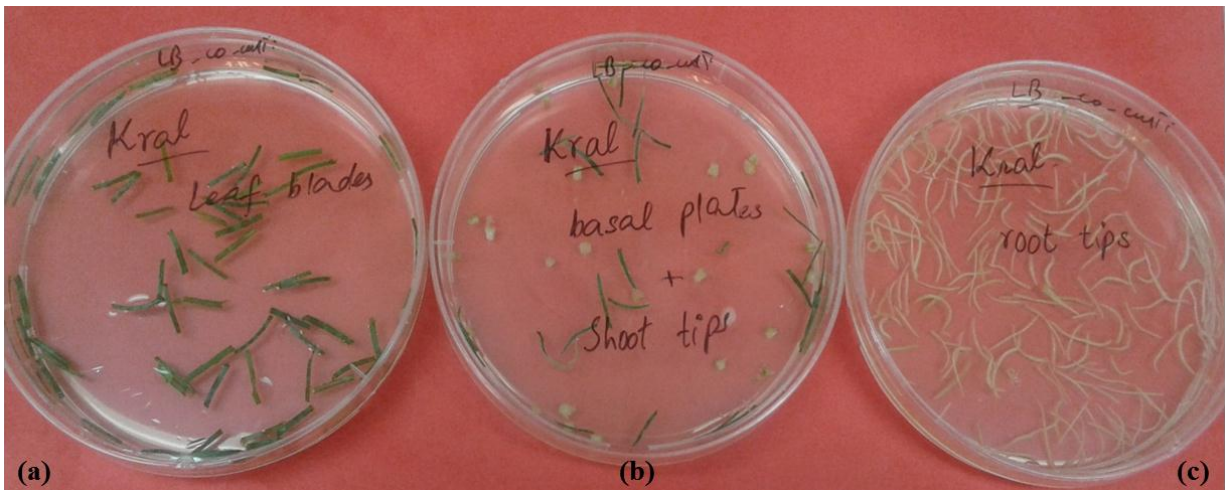


Figure 4.7. Inoculation of onion explants with *Agrobacterium* suspension, (a) leaf blades of Kral dipped in *Agrobacterium* suspension, (b) basal plates and shoot tips of Kral subjected to inoculation with *Agrobacterium*, (c) inoculation of Kral root tips with *Agrobacterium.*

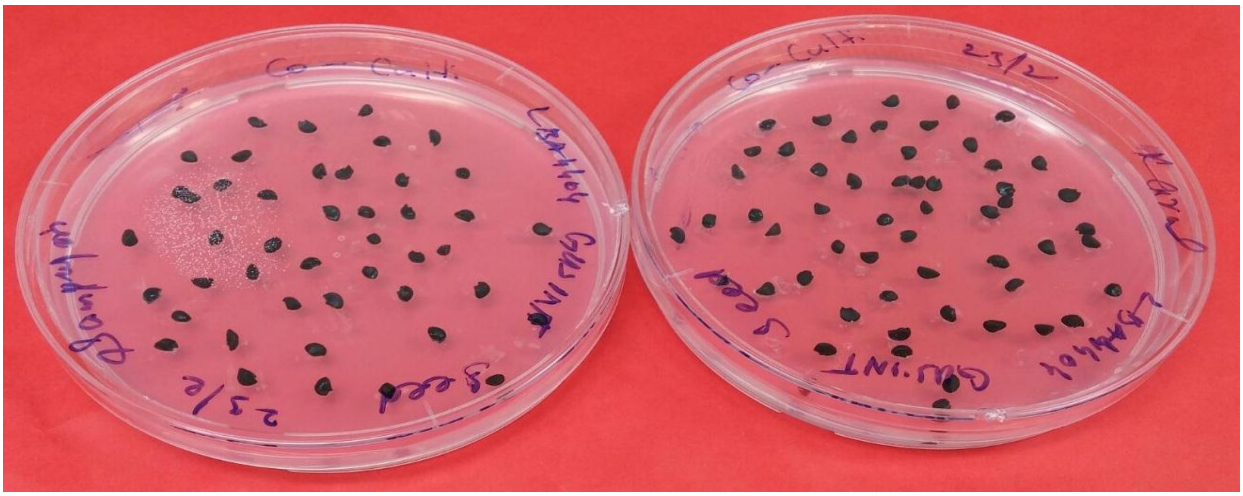


Figure 4.8. Seeds of Sampiyon and Kral on co-cultivation medium after inoculating with *Agrobacterium* (LBA4404) suspension.

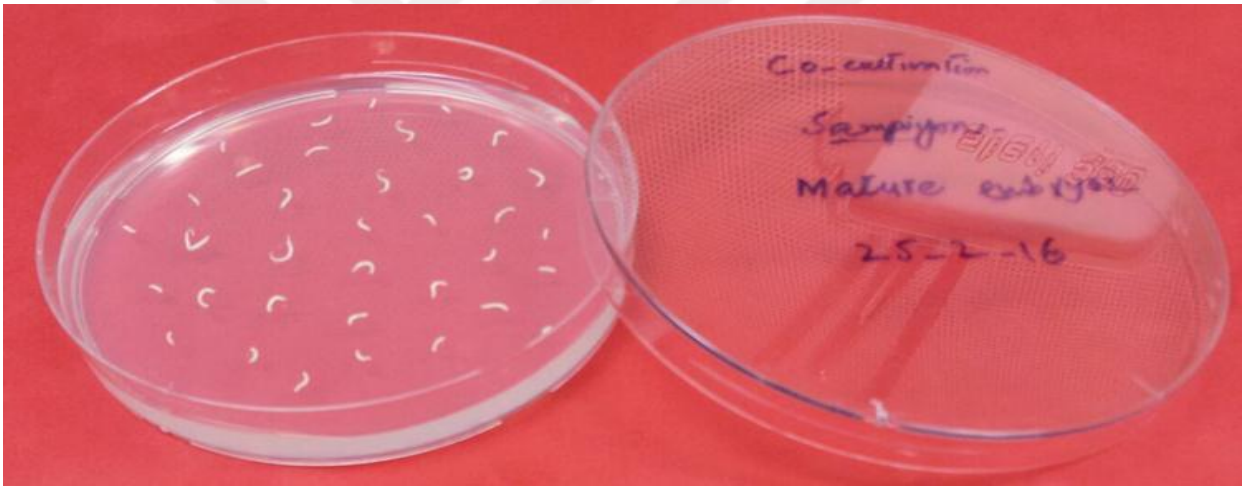


Figure 4.9. Mature embryos of Sampiyon on co-cultivation medium after inoculating with *Agrobacterium*(LBA4404) suspension.

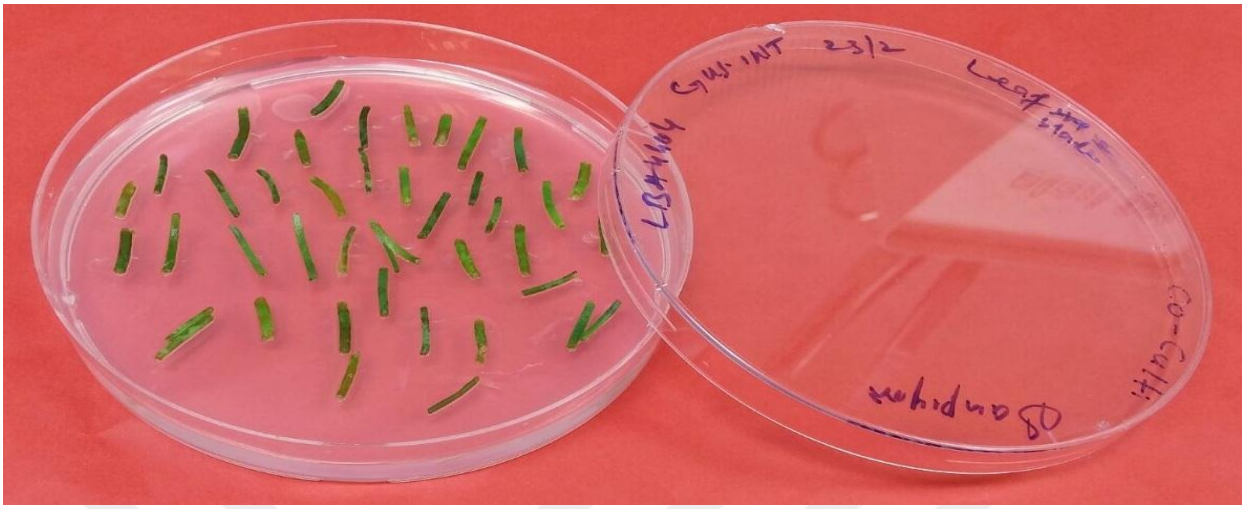


Figure 4.10. Leaf blades of Sampiyon on co-cultivation medium after inoculating with *Agrobacterium* (LBA4404) suspension.

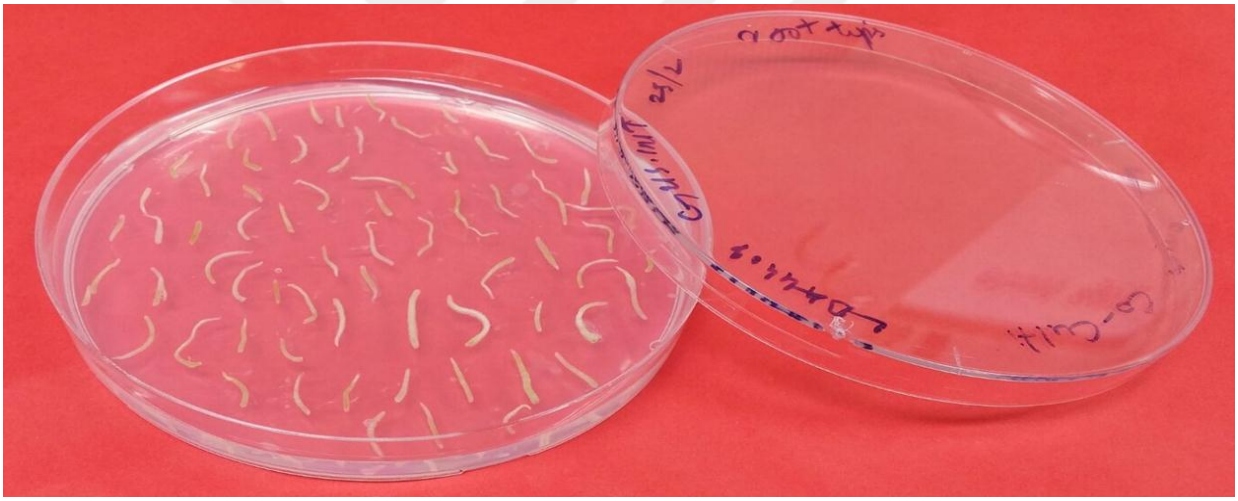


Figure 4.11. Root tips of Sampiyon on co-cultivation medium after inoculating with *Agrobacterium* (LBA4404) suspension.

4.4 Response of Cultivars on Regeneration Selection Media

Three types of regeneration selection media were tested to check the effect of different growth regulators on in-vitro onion regeneration. The growth regulators were used in different combinations. The response of each explant was different on RSM even it varied on different regeneration selection medium.

On RSM-1, regeneration was observed in mature embryos and seeds in both onion cultivars. Basal plates of both onion cultivars showed callus formation and direct regeneration. Shoot tips and root tips did not show any response on RSM-1, while dark green calli were observed in leaf blades of both onion cultivars (Table 4.2). On RSM-2, the explants showed higher frequency of callus induction as compared to RSM-1. Mature embryos and basal plates showed callus induction on this medium. Root tips also showed callus induction. Direct regeneration was observed in seeds of both onion cultivars. Shoot tips of Kral and Sampiyon did not show any response. Dark green calli were observed in leaf blades (Table 4.3). On RSM-3, the frequency of callus induction was very less. Regeneration was observed in basal plates, seeds and mature embryos. Shoot tips, root tips and leaf blades did not show any response (Table 4.4).

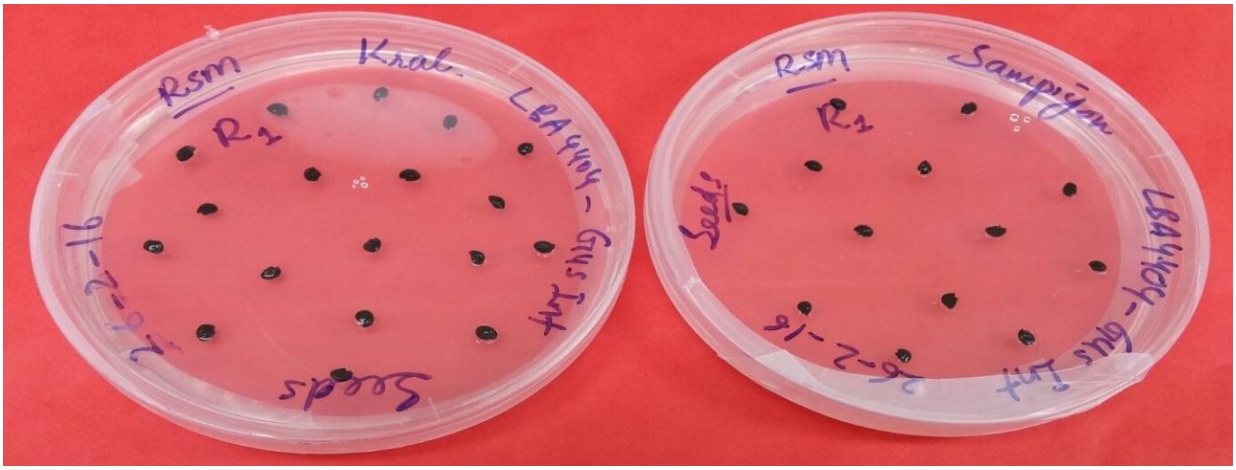


Figure 4.12. Placement of onion seeds on RSM after three days of co-cultivation.

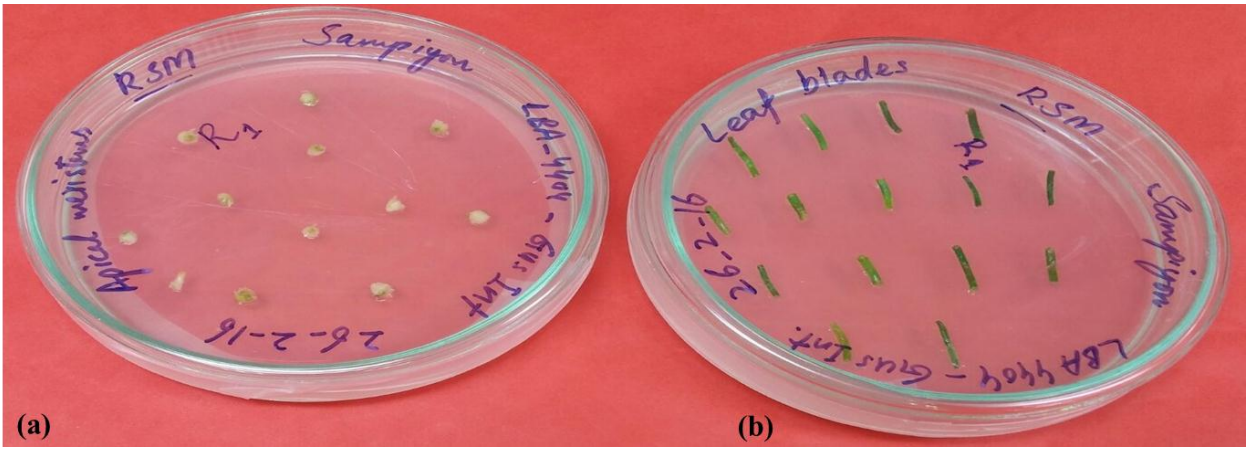


Figure 4.13. Onion basal plates (a) and lead blades (b) on RSM.

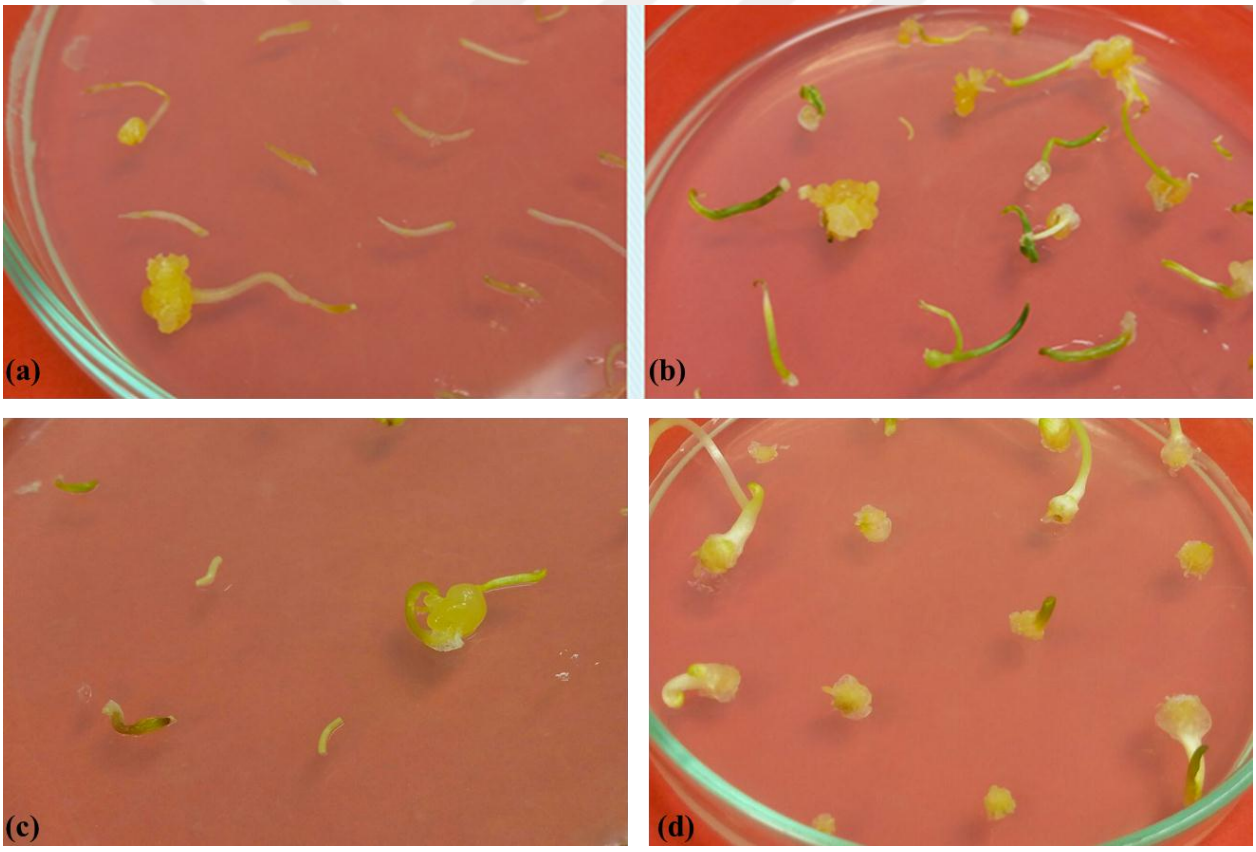


Figure 4.14. Callus formation in roots (a) and mature embryos (b and c) after 3 weeks on RSM, basal plates showing callus induction and regeneration (d).

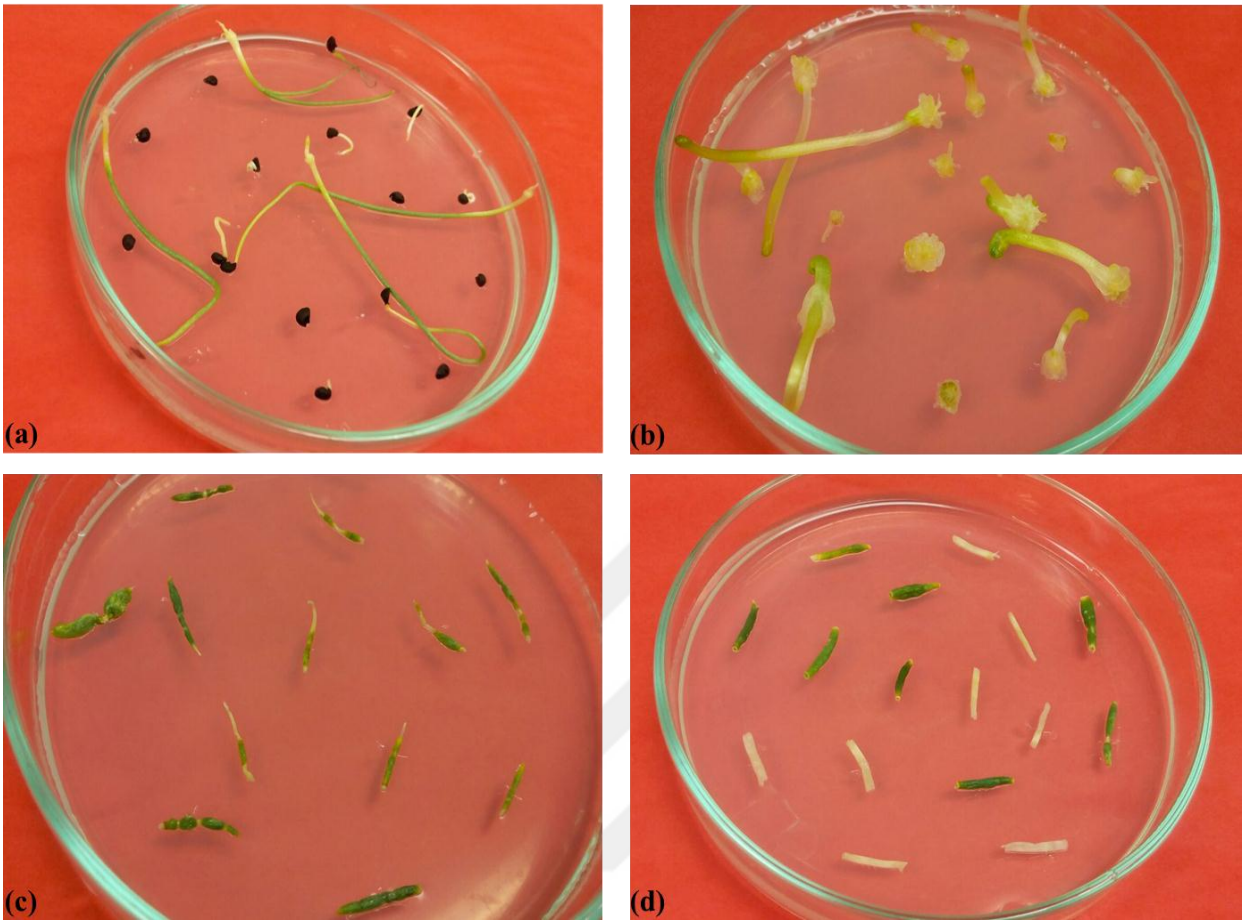


Figure 4.15. Regeneration in onion seeds on RSM (a), basal plates showing regeneration (b), response of shoot tips (c) and leaf blades (d) on RSM.

Table 4.2. Response of Explants on RSM-1 and Shoot/Root Media

Variety	Explants used	Total # of Explants	Callus induction and Proliferation %	Direct Regeneration %	Shoot/Root Medium	Transferred to soil	PCR positive Plants	Transformation Efficiency %
Sampiyon	Basal Plates	90	24.4(0)	16.22	13	9	1	1.1
	Mature Embryos	90	0	93.3	84	49	8	9
	Seeds	90	0	45.6	41	25	9	10
	Shoot Tips	90	18.9*(0)	0	0	0	0	0
	Root Tips	90	0	0	0	0	0	0
	Leaf Blade	90	77.8*(0)	0	0	0	0	0
Kral	Basal Plates	90	8.9(0)	21	17	12	7	8
	Mature Embryos	90	0	60	54	22	15	17
	Seeds	90	0	47.8	43	30	11	12
	Shoot Tips	90	20*(0)	0	0	0	0	0
	Root Tips	90	0	0	0	0	0	0
	Leaf Blade	90	44.4*(0)	0	0	0	0	0

The * symbol is indicating Dark green callus.
RSM-1 = MS basal medium supplemented with 2 mg/l BAP, 0.2 mg/l NAA.
Shoot/Root medium =MS medium supplemented with 2 mg/l BAP, 0.2 mg/l NAA, 1 mg/lGA3.

Table 4.3. Response of Explants on RSM-2 and Shoot/Root Media

Variety	Explants used	Total # of Explants	Callus induction and Proliferation %	Direct Regeneration %	Shoot/Root Medium	Transferred to soil	PCR positive Plants	Transformation Efficiency %
Sampiyon	Basal Plates	90	67.8(0)	25	21	18	4	4.4
	Mature Embryos	90	35.6(0)	42.2	35	18	4	4.4
	Seeds	90	0	90	81	35	6	7
	Shoot Tips	90	0	0	0	0	0	0
	Root Tips	90	17.8(0)	0	0	0	0	0
	Leaf Blade	90	32.2*(0)	0	0	0	0	0
Kral	Basal Plates	90	68.9(0)	27.8	25	12	6	7
	Mature Embryos	90	40.6(0)	55.1	48	23	4	4.4
	Seeds	90	0	86.7	78	23	7	8
	Shoot Tips	90	0	0	0	0	0	0
	Root Tips	90	13.3(0)	0	0	0	0	0
	Leaf Blade	90	52.2*(0)	0	0	0	0	0

The * symbol is indicating Dark green callus.
RSM-2 = MS basal medium supplemented with 0.05 mg/l BAP, 0.5 mg/l 2,4-D.
Shoot/Root medium =MS medium supplemented with 2 mg/l BAP, 0.2 mg/l NAA, 1 mg/lGA3.

Table 4.4. Response of Explants on RSM-3 and Shoot/Root Media

Variety	Explants used	Total # of Explants	Callus induction and proliferation %	Direct Regeneration %	Shoot/Root medium	Transferred to soil	PCR positive Plants	Transformation Efficiency %
Sampiyon	Basal Plates	90	0	90	81	10	3	3.3
	Mature Embryos	90	0	94.4	85	4	0	0
	Seeds	90	0	87.8	79	18	1	1.1
	Shoot Tips	90	0	0	0	0	0	0
	Root Tips	90	7.7(0)	0	0	0	0	0
	Leaf Blade	90	0	0	0	0	0	0
Kral	Basal Plates	90	10(0)	82.2	73	17	0	0
	Mature Embryos	90	0	80	72	6	0	0
	Seeds	90	0	87.8	79	24	1	1.1
	Shoot Tips	90	0	0	0	0	0	0
	Root Tips	90	0	0	0	0	0	0
	Leaf Blade	90	0	0	0	0	0	0

RSM-3= MS basal medium supplemented with 2 mg/l BAP, 2 mg/l NAA.
Shoot/Root medium = MS medium supplemented with 2 mg/l BAP, 0.2 mg/l NAA, 1 mg/lGA3.

4.5 Transfer to Shoot/Root medium

Well-developed onion micro-shoots (obtained from regeneration medium) were transferred to shooting and rooting medium to develop in-vitro transgenic plants (Figure 4.16). No any problem of rooting in primary transformants was recorded. Shoot elongation media also resulted in robust rooting in both cultivars.

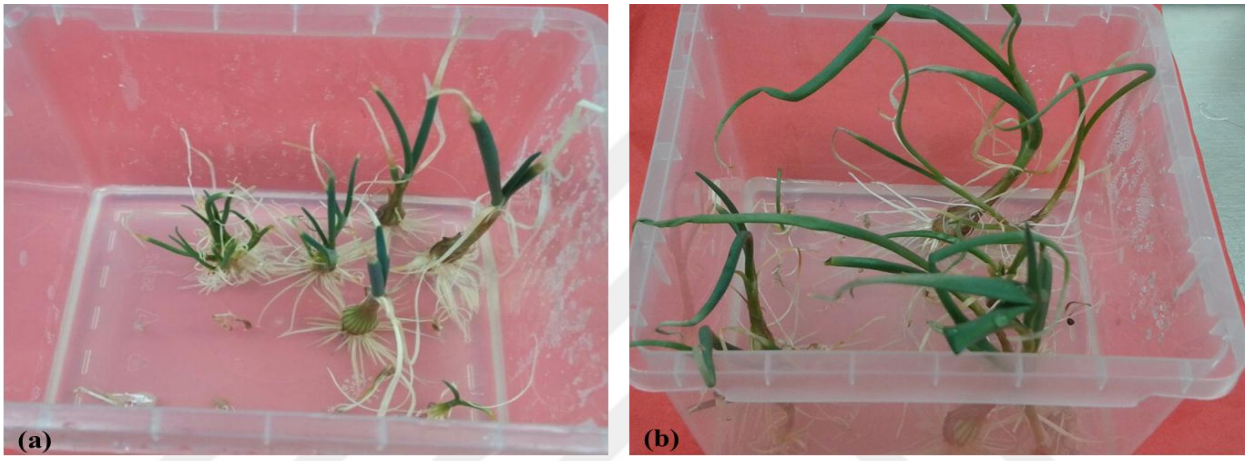


Figure 4.16. In-vitro putative transgenic plants of Sampiyon and Kral respectively.

4.6 Acclimatization

In-vitro onion putative transgenic plants were transferred to soil and kept in green house to acclimatize them in soil conditions (Figure 4.17).

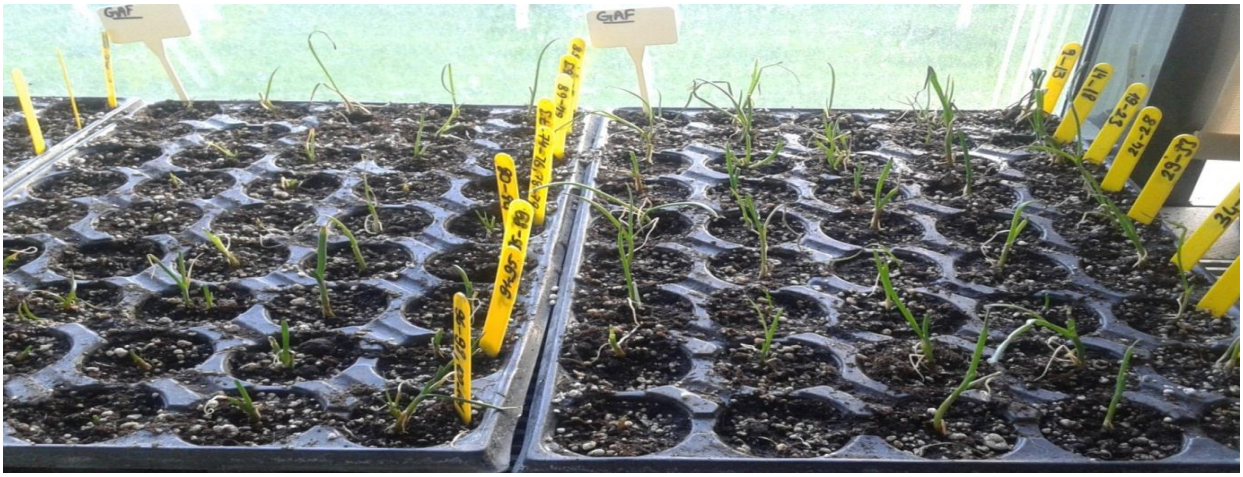


Figure 4.17. Transfer of onion putative transgenic plants in soil.

4.7 GUS Histochemical Assay

When the regenerated shoots were dipped in X-Gluc solution in an eppendorf and kept at 37^0C for one hour to overnight, they turned blue due to GUS activity ensuring transformed cells. The photographs were taken using microscope facility in nematology lab of the faculty. However, the final results were recorded after PCR confirmation. All explants of Kral showed GUS staining except root tips. The GUS expression in root tips was very less.

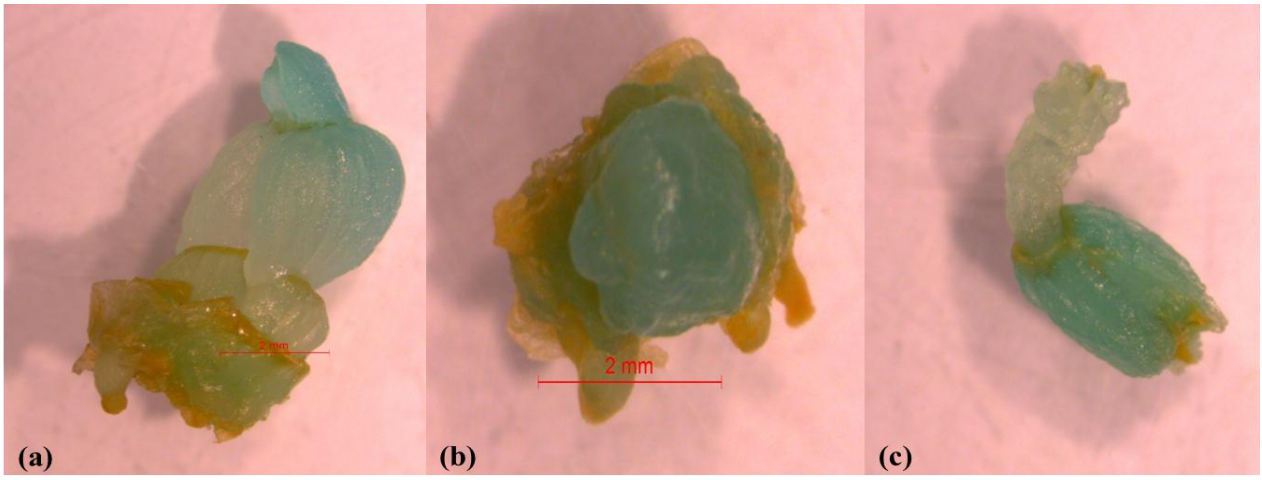


Figure 4.18. GUS staining in basal plates of Kral. (a and c) GUS staining in shoots emerging from basal plates, (b) GUS staining in basal plate callus.

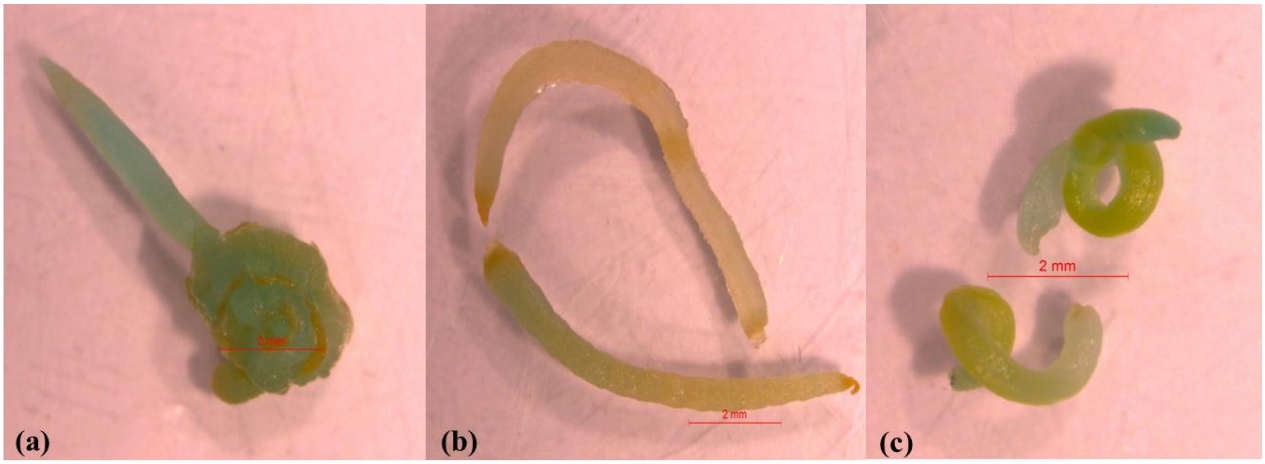


Figure 4.19. GUS staining in basal plates, root tips and mature embryos of Kral. (a) root emerging from basal plate showing GUS activity, (b) GUS expression in root tips, (c) GUS expression in mature embryos.

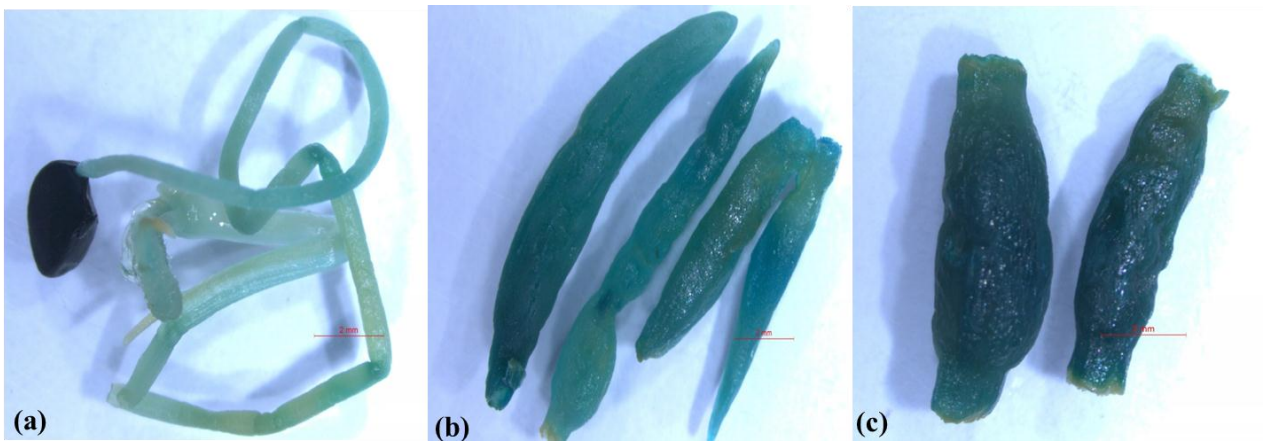


Figure 4.20. GUS staining in seedlings, shoot tips and leaf blades of Kral. (a) seedling emerging from seed showing blue staining, (b) GUS expression in shoot tips, (c) GUS expression in swollen dark green calli of leaf blades.

All explants of Sampiyon showed GUS staining except root tips. The GUS expression in root tips was very less. Shoots emerging from basal plates shoot tips and lead blades showed good GUS activity.

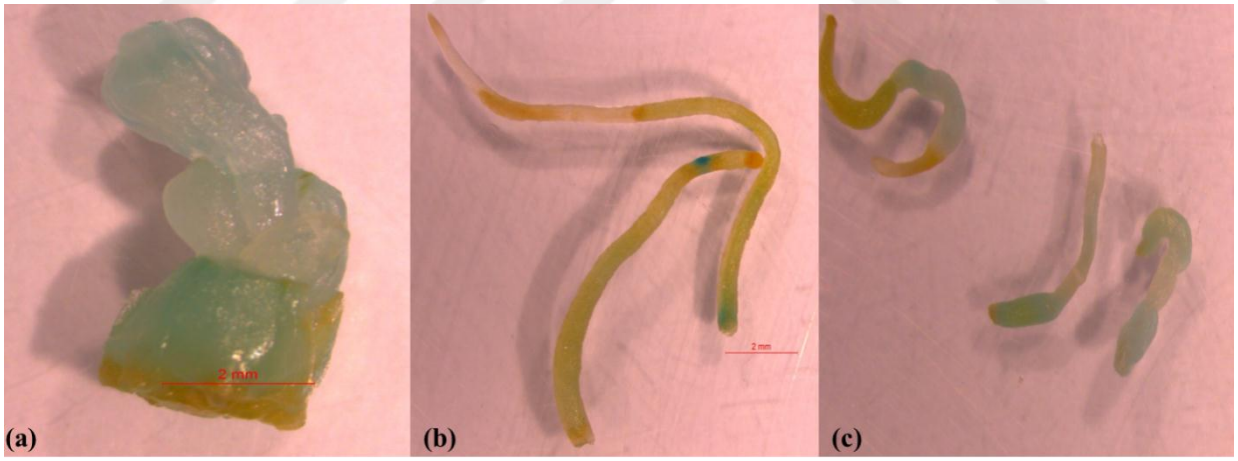


Figure 4.21. GUS staining in basal plate, root tips and mature embryos of Sampiyon. (a) shoot emerging from basal plate showing blue staining, (b) GUS expression in root tips, (c) GUS expression in mature embryos.

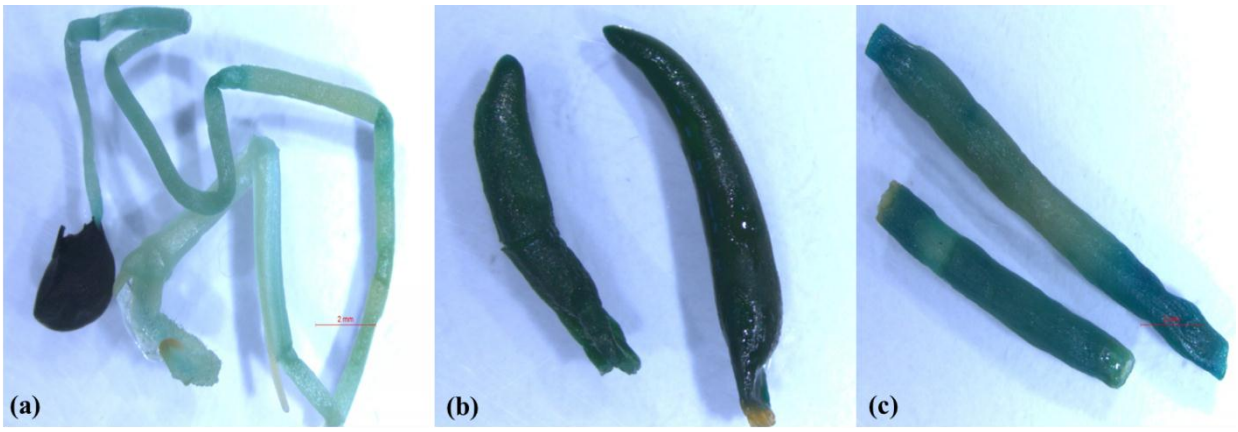


Figure 4.22. GUS staining in seedlings, shoot tips and leaf blades of Sampiyon. (a) seedling emerging from seed showing blue staining, (b) GUS expression in shoot tips, (c) GUS expression in leaf blades.

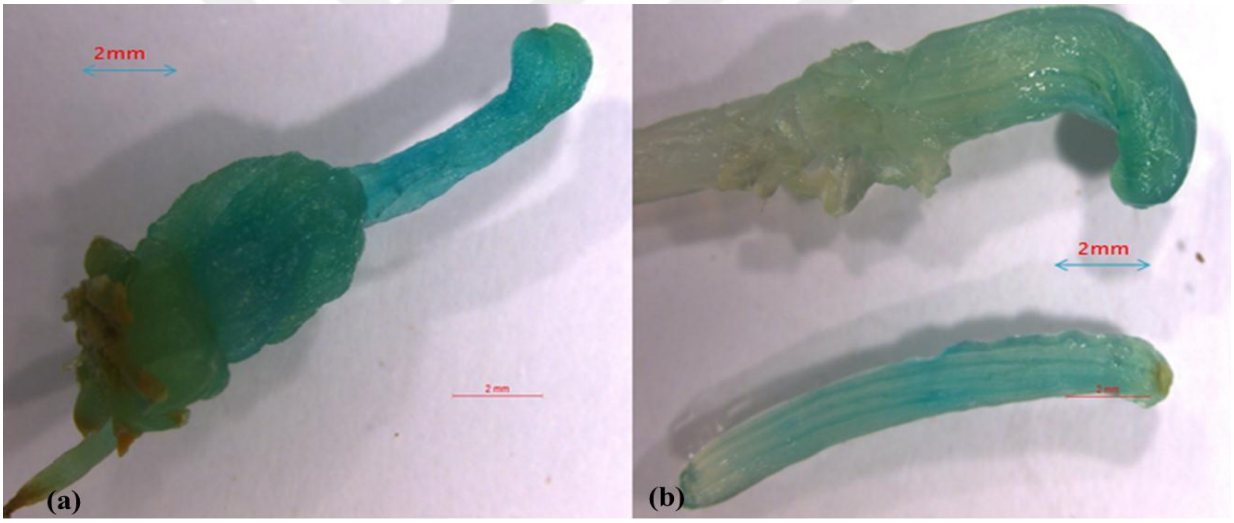


Figure 4.23. GUS staining in shoots emerging from basal plates. Sampiyon (a) and Kral (b).

4.8 Confirmation of Transgene through PCR

In order to confirm presence of introduced genes (*nptII* and *uidA* genes) in primary transformants, PCR analysis was done of total 355 plant samples. Plant samples were named from 501 to 855. All positive samples were screened for transgene followed by reconfirmation of screened samples through PCR and gel electrophoresis (see in Appendices). 87 plant samples gave positive PCR results confirming that presence of transgene in plant genome (Table 4.5). Transgenic plants showed 450 bp band size (for *nptII* gene) and 362 bp band size (for *uidA* gene) on agarose gel. The plasmid DNA was used as positive controls. For wild control, non-transformed DNA "B17-50B" (provided by GAF) was used.

Table 4.5. Arrangement of Positive Samples on PCR Plate

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	A (+)	550	580	593	602	615	627	664	707	730	766	854
B	B (-)	552	581	594	603	616	631	676	709	733	767	Wild
C	502	554	582	595	604	618	632	680	716	743	768	
D	522	559	583	596	605	619	637	682	720	746	771	
E	525	560	584	597	606	620	638	683	721	753	779	
F	527	576	586	598	607	621	656	685	722	754	781	
G	529	577	590	600	608	622	659	688	723	755	782	
H	541	579	592	601	612	623	663	689	726	756	805	

Table 4.6. GUS Assay and PCR confirmation of Positive Samples

Plant No.	Assigned No.	GUS Assay	*nptII* gene positive	*uidA* gene positive
1	502	+	+	+
2	522	+	+	+
3	525	+	+	+
4	527	+	+	+
5	529	+	+	+
6	541	+	+	+
7	550	+	+	+
8	552	+	+	+
9	554	+	+	+
10	559	+	+	+
11	560	+	+	+
12	576	+	+	+
13	577	+	+	+
14	579	+	+	+
15	580	+	+	+
16	581	+	+	+
17	582	+	+	+
18	583	+	+	+
19	584	+	+	+
20	586	+	+	+
21	590	+	+	+
22	592	+	+	+
23	593	+	+	+
24	594	+	+	+
25	595	+	+	+
26	596	+	+	+
27	597	+	+	+
28	598	+	+	+
29	600	+	+	+
30	601	+	+	+

Table 4.6. (Continue) GUS Assay and PCR confirmation of Positive Samples

Plant No.	Assigned No.	GUS Assay	*nptII* gene positive	*uidA* gene positive
31	602	+	+	+
32	603	+	+	+
33	604	+	+	-
34	605	+	+	+
35	606	+	+	+
36	607	+	+	+
37	608	+	+	+
38	612	+	+	+
39	615	+	+	+
40	616	+	+	+
41	618	+	+	+
42	619	+	+	+
43	620	+	+	+
44	621	+	+	+
45	622	+	+	+
46	623	+	+	+
47	627	+	+	+
48	631	+	+	+
49	632	+	+	+
50	637	+	+	+
51	638	+	+	+
52	656	+	+	+
53	659	+	+	+
54	663	+	+	+
55	664	+	+	+
56	676	+	+	+
57	680	+	+	+
58	682	+	+	+
59	683	+	+	+
60	685	+	+	+

Table 4.6. (Continue) GUS Assay and PCR confirmation of Positive Samples

Plant No.	Assigned No.	GUS Assay	*nptII* gene positive	*uidA* gene positive
61	688	+	+	+
62	689	+	+	+
63	707	+	+	+
64	709	+	+	+
65	716	+	+	+
66	720	+	+	+
67	721	+	+	+
68	722	+	+	+
69	723	+	+	+
70	726	+	+	+
71	730	+	+	+
72	733	+	+	+
73	743	+	+	+
74	746	+	+	+
75	753	+	+	+
76	754	+	+	+
77	755	+	+	+
78	756	+	+	+
79	766	+	+	+
80	767	+	+	+
81	768	+	+	+
82	771	+	+	+
83	779	+	+	-
84	781	+	+	+
85	782	+	+	+
86	805	+	+	+
87	854	+	+	+

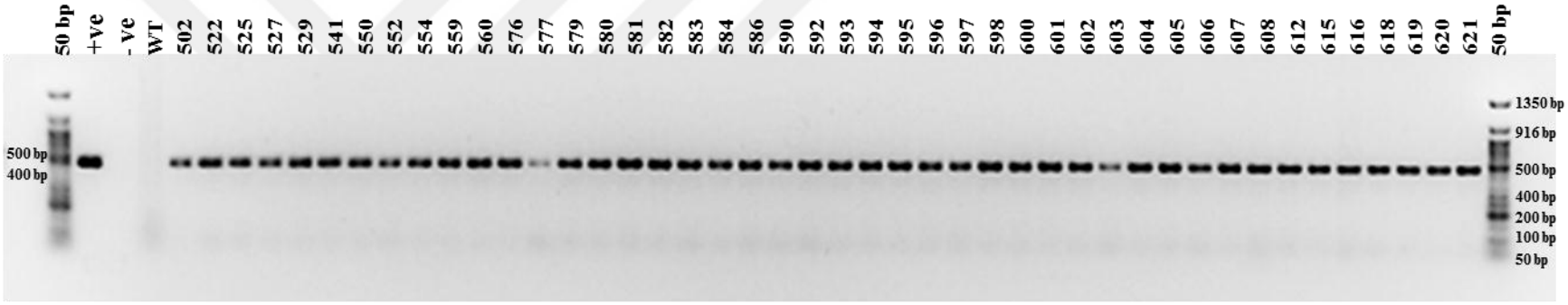


Figure 4.24. PCR confirmation for the presence of 450 bp fragment of *nptII* gene, 50 bp DNA Ladder; **+ve =** positive control (pRD400 Plasmid); **-ve** = without DNA control; **WT=** wild non-transformed DNA; Lane 502-621 putative transgenic plants of Sampiyon and Kral cultivars

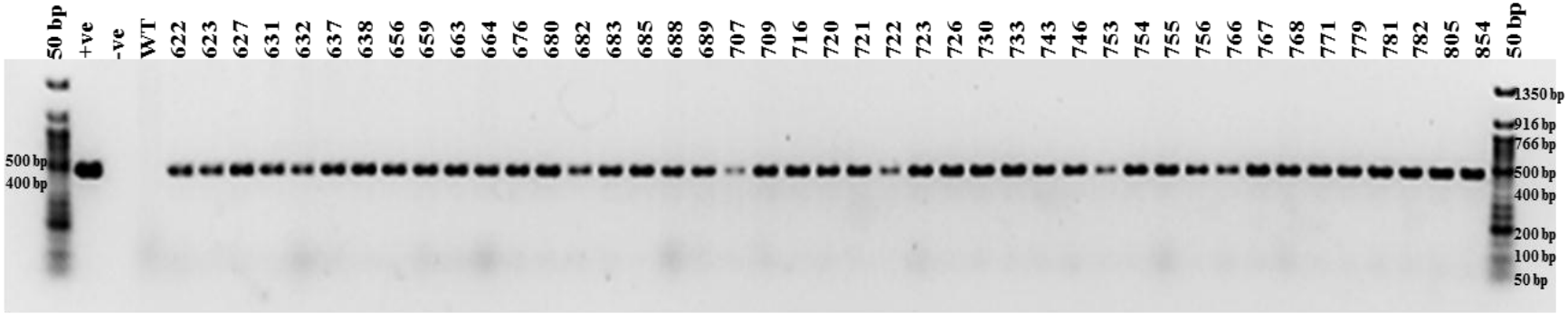


Figure 4.25. PCR confirmation for the presence of 450 bp fragment of *nptII* gene, 50 bp DNA Ladder; **+ve =** positive control (pRD400 Plasmid); **-ve**= without DNA control; **WT=** wild non-transformed DNA, Lane 622-854 putative transgenic plants of Sampiyon and Kral cultivars

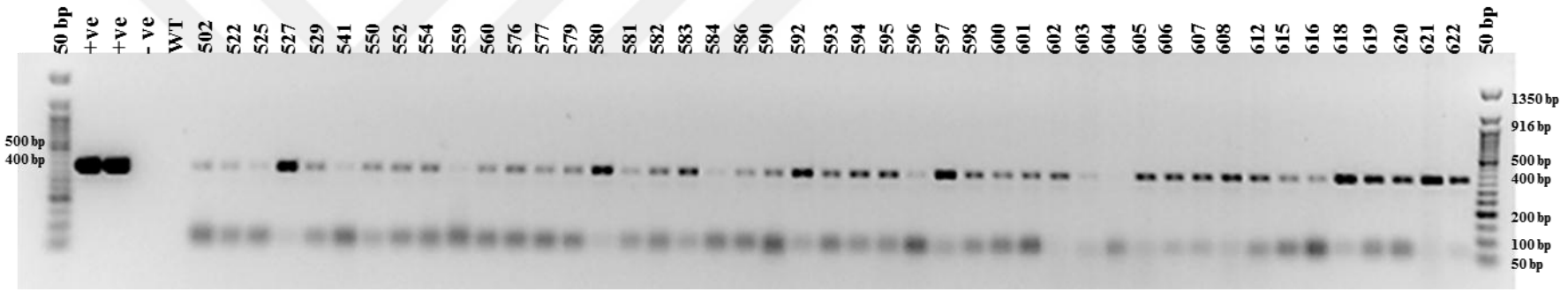


Figure 4.26: PCR confirmation for the presence of 362 bp fragment of *uidA* gene, 50bp DNA Ladder; **+ve (i) =** positive control (pCambia Plasmid); **+ve (ii)** = positive control (pCam-EPSPS Plasmid); **-ve** = without DNA control; **WT=** wild non-transformed DNA; Lane 502-622 putative transgenic plants of Sampiyon and Kral cultivars

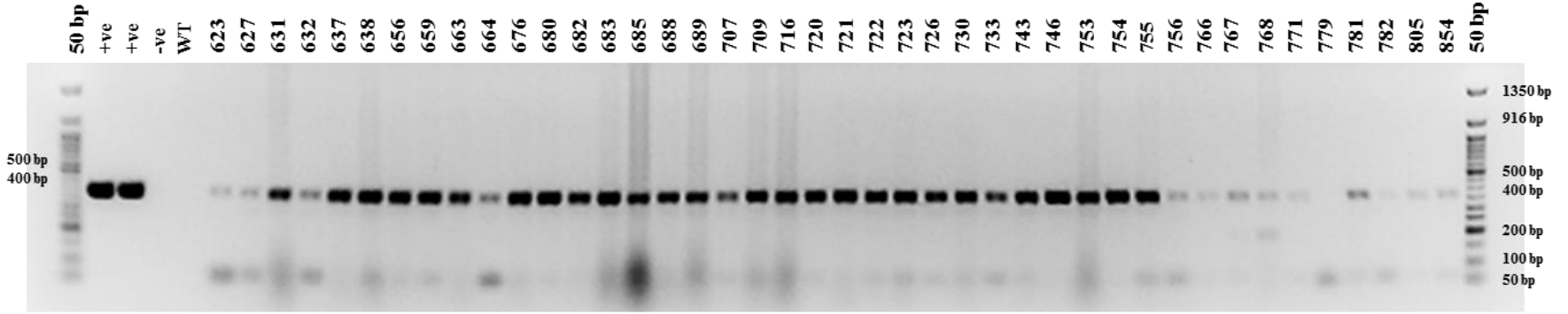


Figure 4.27: PCR confirmation for the presence of 362 bp fragment of *uidA* gene, 50bp DNA Ladder; **+ve (i) =** positive control (pCambia Plasmid); **+ve (ii)** = positive control (pCam-EPSPS Plasmid); **-ve** = without DNA control; **WT=** wild non-transformed DNA; Lane 623-854 putative transgenic plants of Sampiyon and Kral cultivars

4.9 Calculation of Transformation Efficiency

Transformation efficiency of each targeted explants for *Agrobacterium* mediated transformation was calculated. Transformation efficiency was different for each explant used for this study; even it varied from medium to medium. Three different types of RSM were used to optimize transformation in-vitro onion transformation and regeneration efficiency.

In the total PCR positive plants obtained, highest frequency of transgenic plants was contributed by mature embryos followed by seeds and basal plates (Table 4.2, 4.3 and 4.4). None of positive transgenic plant was obtained from shoot tips, root tips and leaf blades in the transformation experiments performed. Basal plates showed good transformation efficiencies on RSM-1 and RSM-2. Mean transformation frequency for basal plates on RSM-1 was 1.1 % for Sampiyon and 8 % for Kral (Table 4.2). Mean transformation frequency for basal plates on RSM-2 was 4.4 % for Sampiyon and 7 % for Kral (Table 4.3). On RSM-3, transformation ability of basal plates was zero in Kral and in case of Sampiyon; the mean transformation frequency was 3.3 %. In the case of genotype effect, basal plates of both onion cultivars gave good transformation efficiencies on RSM-1 and RSM-2, however, in case of RSM-3, Sampiyon performed well (Table 4.4). On other two RSM, Kral was observed performing well overall.

Mature embryos showed good regeneration on RSM-1 and RSM-2. Mean transformation frequency for mature embryos on RSM-1 was observed 9 % for Sampiyon and 17 % for Kral (Table 4.2). Mean transformation frequency for mature embryos on RSM-2 was 4.4 % for both cultivars (Table 4.3). In case of genotype differences on media, Kral was better on RSM-1 with 17 % transformation efficiency. On RSM-3, the transformation efficiency was zero.

Seeds of both onion breeding lines performed well on all regeneration media. However, good transformation and regeneration efficiencies were observed on RSM-1 and RSM-2. Mean transformation frequency for seeds on RSM-1 was observed 10 % for Sampiyon and 12 % for Kral (Table 4.2). Mean transformation frequency for seeds on RSM-2 was 7 % for Sampiyon and 8 % for Kral (Table 4.3). Kral performed well as compared to Sampiyon on both RSM media. On RSM-3, transformation efficiencies were not as good as on RSM-1 and RSM-2.

Shoot tips, root tips and leaf blades did not show any response on regeneration media. None of positive transgenic plant was obtained from them (Table 4.2, 4.3 and 4.4). For each medium, genotype efficiency for transformation was different. However, in this study, Kral performed well overall. In the total of 87 onion transgenic plants, 51 contributed by Kral and 36 obtained from Sampiyon. The highest transformation frequency obtained was 17 % in mature embryos followed by 12 % in seeds and 8 % in basal plates.

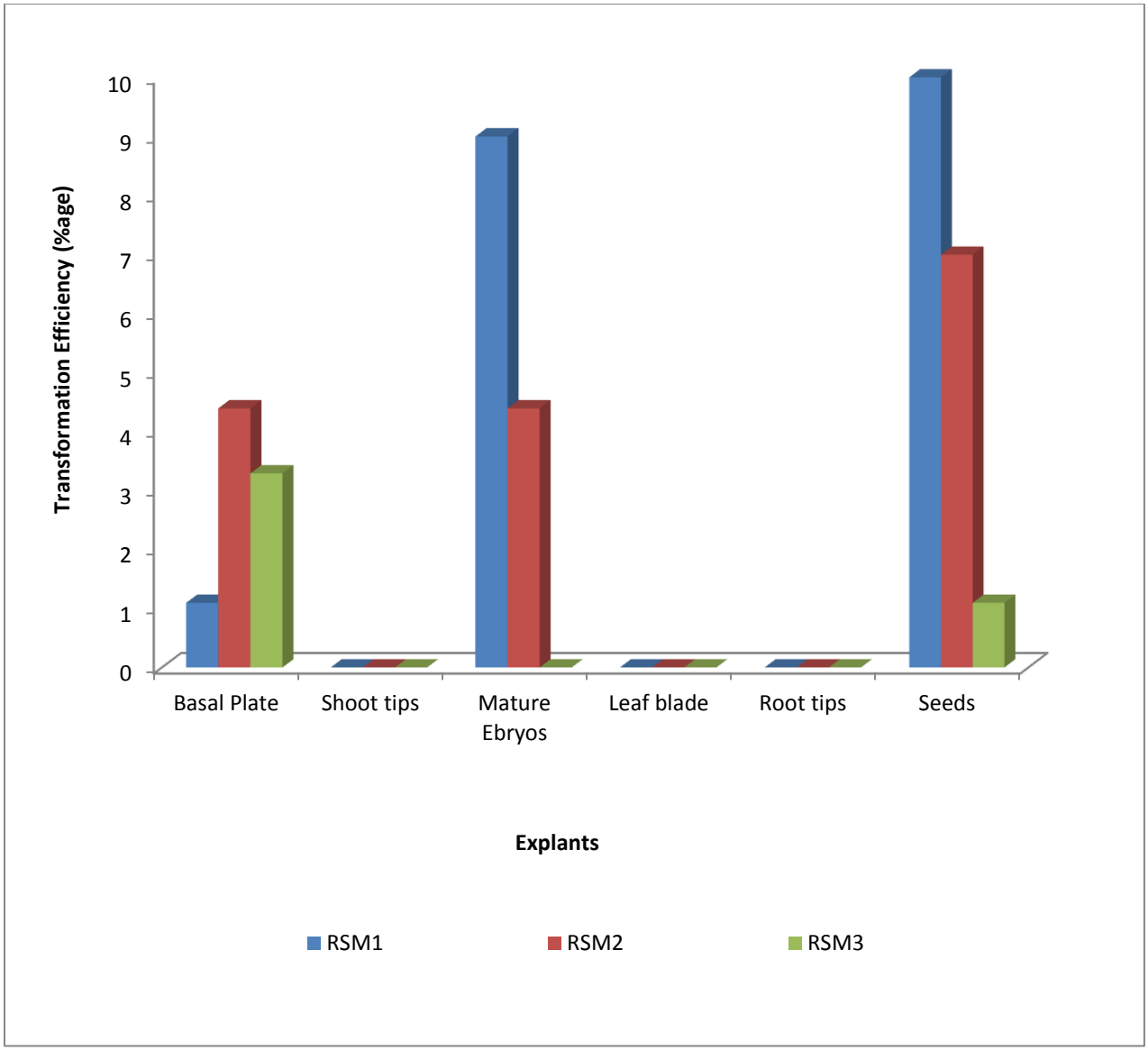


Figure 4.28. Transformation Efficiency in Sampiyon cultivar using different explants.

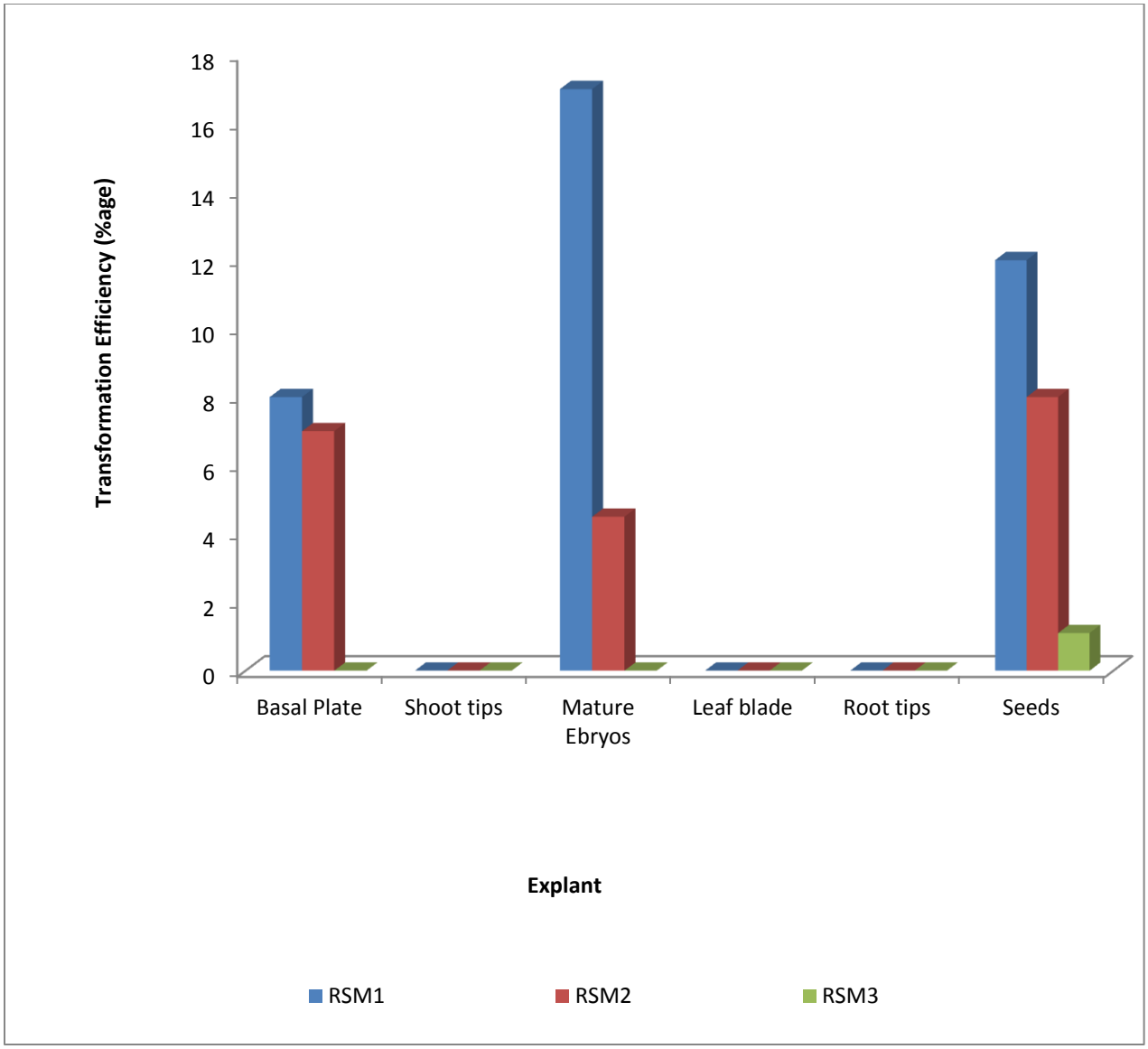


Figure 4.29. Transformation Efficiency in Kral cultivar using different explants.

CHAPTER V

DISCUSSION

5.1 Response of Different Onion Cultivars

Onion being a monocotyledonous crop was perceived to be a difficult crop for genetic transformation (Arumuganathan and Earle, 1991). Onion having large genome size poses less response to transform genetically which may reduce the likelihood of transgene integration at active site of genome and probability to have correct transgene expression may affect (Eady et al., 1995a). Due to less response of monocots for *Agrobacterium*-mediated transformation, particle bombardment method was used to transform monocotyledonous crops genetically (Klein et al., 1987 and Christou et al., 1992).

An optimized and functionally stable protocol having a reasonable number of transformants may solve the problem of less responsiveness of monocots for *Agrobacterium*-mediated transformation (Arumuganathan and Earle, 1991). Many reports on successful *Agrobacterium*-mediated transformation in cereals have been published. Wheat, rice, sorghum and barley are among those monocot plants that can be efficiently transformed by *Agrobacterium tumefaciens* (Hiei et al., 1994; Hiei et al., 1997; Ozawa, 2012 and Hiei et al., 2014). Present investigation was planned to optimize the factors influencing *Agrobacterium*-mediated transformation efficiency in onion. The plant transformation and regeneration frequency is influenced by genotype, explants and the composition of regeneration media as well as interaction between genotype and media used (Khanna and Raina, 1998). Nine onion cultivars were suggested to test their in-vitro germination response. The seeds were grown on MS basal medium. Out of 9 different verities tested, Sampiyon and Kral gave good in-vitro germination %. These two cultivars were subjected to transformation experiments.

Different onion genotypes responded differently on regeneration medium. There were large differences in their response to regeneration system (Valk et al., 1992). Several other reports favored the similar results about response of different genotypes on regeneration media (Kamstaityte et al. 2004; Khar et al., 2005 and Hailekidan et al., 2013). Phillips and

Hubstenberger (1987) reported that the hybrids of *Allium fistulosum* x *Allium cepa* were highly responsive to regeneration system. Furthermore, Eady et al. (1995b) documented that "Yellow Express" also known as "Sapporo Yellow" onion cultivars gave relatively best response to in-vitro experiments.

The current research investigated that onion transformation and in-vitro regeneration system were dependent on the cultivar and explants used. Results showed that the variety "Kral" performed better considering the response of callus induction and further regeneration. GUS expression was good in Kral as compared to Sampiyon. Out of 87 total onion transgenic plants obtained, 51 were contributed by Kral. Buitveld and Creemers-Molenaar (1994) documented the similar results about genotype and explants dependency in leek transformation and regeneration cultures. The putative transgenic plants were subjected to PCR analysis to confirm the presence of *nptII* gene.87 positive transgenic plants were obtained out of total 355 regenerated plants. Results showed amplification of 450 bp and 362 bp product size for *nptII* gene and *uidA* gene respectively, indicating the proper incorporated transgene in onion genome (Figure 4.24 and 4.25). A notable number of transgenic escapes were found at this stage suggesting that selection system was not effective enough.

Zheng et al., (2001) reported the similar phenomenon that a number of untransformed plants escaped from selection in onion transformation experiments with three-week old callus, induced from mature embryos, as target tissues using *Agrobacterium* and hygromycin as a selection system. They reported that selection on hygromycin resistance was not effective in this case. Chopping of callus to allow more exposure to the selective agent during subculture in different selective media might reduce these escapes.

5.2 Response of Different Explants

Several initial reports were given on explants dependency on transformation system, callus induction and regeneration cultures (Hussey and Falavigna, 1980; Rabinowitch and Brewster, 1990; Eady et al., 1995; Zheng et al., 1998; Eady et al., 1998a; Eady et al., 1998b; Eady et al., 2000; Zheng et al., 2001; Marinangeli et al. 2005 and Zheng et al., 2005).

In the present study, mature embryos, basal plates and seeds gave good response on callus inducing and regeneration media. The highest transformation frequency obtained was 17 % in mature embryos followed by 12 % in seeds and 8 % in basal plates. Dark green callus observed in shoot tips and leaf blades but these calli did not show response on shoot induction medium. Root tips also showed tendency to make callus on callus inducing medium. None of positive transgenic plant was obtained from shoot tips, root tips and leaf blades.

Mature embryos could prove efficient explants in onion transformation and regeneration experiments. Similar findings were documented by Buitveld et al. (1994), Valk et al. (1992), and Shahin and Kaneko (1986) who conducted experiments using onion mature embryos or seedlings derived calli. The callus derived from embryo showed high regeneration frequency. Zheng et al. (1998, 2001 and 2005) documented various successful reports on onion transformation and regeneration using mature embryos as experimental material.

Basal discs or basal plates also showed potential to be an efficient explant for onion transformation and in-vitro regeneration cultures in this study. Hailekidan et al. (2013) and Malla et al. (2015) studied micro-propagation and DNA delivery system in onion. They individually documented similar results by taking basal disc as experimental material. Viterbo et al. (1992) reported that the most efficient callus systems were derived from basal plates and embryos.

The response of seeds in transformation and regeneration was effectual showing good transformation efficiency. From the limited literature, available on *Agrobacterium*-mediated transformation in onion, there is no work done on direct seed transformation of onion. In the current study, sterilized seeds were used as explants in transformation experiments. The seedlings emerged from treated seeds with *Agrobacterium* suspension showed good GUS staining and mean frequency of transformation %. Direct regeneration was observed on regeneration media. The results revealed that full seeds could prove a potential explant for onion transformation experiments due to the round the year availability and easy to work with. The mean transformation efficiency observed in seeds was as good as in mature embryos and basal plates in a specified composition of regeneration media and genotype used.

In GUS histochemical assay, all explants except root tips (basal plates, mature embryos, seeds, shoot tips and leaf blades) showed good GUS activity. Root tips showed little GUS staining. In the total PCR positive plants obtained, highest frequency of transgenic plants was contributed by mature embryos followed by seeds and basal plates. None of positive transgenic plant was obtained from shoot tips, root tips and leaf blades in the transformation experiments performed.

5.3 Effect of Different Plant Growth Regulators on Onion Regeneration

Although plant genotype and type of explant are considered as the major determinants of in-vitro plant regeneration response, as well as composition of callus inducing medium and regeneration medium is another important factor to deal with. An overall analysis of in-vitro response variations also revealed that there was a significant interaction between the callus inducing medium used for de-differentiation and regeneration selection medium used for re-differentiation (Khanna and Raina, 1998). Current study also found out that composition of callus inducing and regeneration media contributed a vital role in successful in-vitro onion regeneration and there was a significant effect of genotype and explants interaction with media used for plant proliferation.

The onion culture requirements of media and plant growth regulator for clonal propagation are not definite. Different researchers used different sources of onion explants, genotypes, experimental conditions and methods of measuring results. Therefore, comparison between different treatments usually may not be possible (Eady, 1995).The outcomes of this study revealed that very high concentrations showed negative effects on callus inducing ability. Similar to these findings were presented by Kamstaityteet al. (2004).

Present research demonstrated that good callus induction proportion was observed in both onion cultivars (Kral and Sampiyon) when MS medium supplemented with BAP and 2,4-D. The best callus induction frequencies were obtained on MS medium supplemented with 0.05 mg/l BAP, 0.5 mg/l 2,4-D. When the concentrations of BAP and 2,4-D in medium were increased from 1:10 ratio respectively, it had negative effect on callus induction. Similar type of results and response of growth regulators has been investigated by Ramakrishnan et al. (2013). Zheng et al. (1998)

also stated that concentration of 2,4-D was the most important factor to optimize to obtain good callus induction and plant regeneration in onion.

Direct regeneration was observed in mature embryos, seeds and basal plates on regeneration medium-1 (MS medium supplemented with 2 mg/l BAP, 0.2 mg/l NAA). When BAP and NAA were used in equal concentrations, mature embryos, seeds and basal plates showed regeneration ability but mean transformation frequencies % were very low on this medium in both cultivars. Regenerated shoots were transferred to shoot/root medium (MS medium supplemented with 2 mg/l BAP, 0.2 mg/l NAA, 1 mg/l GA3, 500 mg/l). In this research study, kanamycin was chosen as selective agent at a concentration of 100 mg/l. A binary vector harboring *nptII* gene conferring kanamycin resistance and GUS reporter gene was used to identify and select transgenic plants. GUS reporter system gave visual identification of transformed callus due to GUS activity (Xu et al. 2007).

The research also revealed that most onion calli usually showed less response to regeneration. A good proportion of callus induction was observed in mature embryos followed by basal plates. Root tips also showed callus induction ability. But mostly calli did not response on regeneration media. A high proportion of onion cells within a callus were important to obtain good frequency of transformants. Similar results were documented by Rabinowitch and Brewster (1990). The frequency of regeneration was observed more in basal plates and seeds rather than making callus.

CHAPTER VI

CONCLUSION

The systematic study was carried out to optimize *Agrobacterium tumefaciens* mediated transformation in onion. Factors affecting callus induction, propagation and plant regeneration ability in onion were investigated, such as response of genotype, type of explants and composition of different plant growth regulators in callus inducing and regeneration media. A binary vector harboring *nptII* gene coffering kanamycin resistance and GUS reporter gene was used to identify and select transgenic plants.

The results supported the genotype and explants dependency in onion transformation and regeneration cultures. Out of 9 different onion verities tested for in-vitro germination %, Sampiyon and Kral gave good response. The variety "Kral" performed better considering the response on callus inducing and regeneration cultures. GUS expression was good in Kral as compared to Sampiyon. Out of 87 total onion transgenic plants obtained, 51 were contributed by Kral.

Mature embryos, basal plates and seeds gave good response on callus inducing and regeneration media. The highest transformation frequency was observed in mature embryos followed by seeds and basal plates. The calli derived from shoot tips and leaf blades did not show response on regeneration medium. Root tips showed little tendency to make callus on callus inducing medium. In the current study, sterilized seeds were used as explants in transformation experiments. The seedlings emerged from treated seeds with *Agrobacterium* suspension showed good GUS staining and mean frequency of transformation %. The use of onion seeds as explants in transformation experiments is a novel work which has not been reported yet in the scant literature available on onion transformation and in-vitro regeneration.

All explants except root tips (basal plates, mature embryos, seeds, shoot tips and leaf blades) showed good GUS activity. Root tips showed little GUS staining. Molecular analysis (PCR) demonstrated that highest frequency of transgenic plants was contributed by mature embryos

followed by seeds and basal plates. None of positive transgenic plant was obtained from shoot tips, root tips and leaf blades in the transformation experiments performed.

The best callus induction frequencies were observed on MS medium supplemented with 0.05 mg/l BAP, 0.5 mg/l 2,4-D. Direct regeneration was observed in mature embryos, seeds and basal plates on regeneration medium-1 (MS medium supplemented with 2 mg/l BAP, 0.2 mg/l NAA, and 500 mg/l). When BAP and NAA were used in equal concentrations, mature embryos, seeds and basal plates showed regeneration ability but mean transformation frequencies % were very low on this medium in both cultivars. The results documented herein may be helpful for the future research regarding introduction of any desired gene in onion by using this optimized transformation strategy.

REFERENCES

Altpeter, F., Baisakh, N., Beachy, R., Bock, R., Capell, T., Christou, P., Daniell, H., Datta, K., Datta, S., Dix, P.J., Fauquet, C., Huang, N., Kohli, A., Mooibroek, H., Nicholson, L., Nguyen, T.T., Nugent, G., Raemakers, K., Romano, A., Somers, D.A., Stoger, E., Taylor, N. and Visser, R., "Particle bombardment and the genetic enhancement of crops: myths and realities", ***Molecular Breeding***, 15, 305-327, 2005.

Aswath, C., Mo, S., Kim, D. and Park, S. "*Agrobacterium* and biolistic transformation of onion using non-antibiotic selection marker phosphomannose isomerase", ***Plant Cell Rep***, 25, 92–99, 2006.

Aragão, F.J.L., Sarokin, L., Vianna, G.R. and Rech, E.L., "Selection of transgenic meristematic cells utilizing herbicidal molecule results in the recovery of fertile transgenic soybean (*Glycine max* L.) plants at a high frequency", ***Theoretical and applied genetics***, 101, 1-6, 2000.

Arumuganathan, K. and Earle, E.D., "Nuclear DNA content of some important plant species", ***Plant molecular biology reporter***, 9 (3), 208-218, 1991.

Barandiaran, X., Dipietro, A. and Martin, J., "Biolistic transfer and expression of a *uidA* reporter gene in different tissues of *Allium sativum* L.", ***Plant Cell Rep***. 17, 737–741, 1998.

Brewster, J.L., "Onions and other vegetable *Alliums* (1st ed.)", Wallingford, UK, ***CAB International***, 1, 16-20, 1994.

Buitveld, J. and Creemers-Molenaar, J., "Plant regeneration from protoplasts isolated from suspension cultures of leek (*Allium ampeloprasum* L.)", ***Plant science***, 100(2), 203-210, 1994.

Christou, P., "Rice transformation – bombardment", ***Plant Mol Biol***, 35, 197–203, 1997.

Christou, P., "Strategies for variety-independent genetic transformation of important cereals, legumes and woody species utilizing particle bombardment", ***Euphytica***, 85, 13–27, 1995.

Christou, P., Ford, T.L. and Kofron, M., "Rice Genetic Engineering: A Review", ***Trends In Biotechnology***, 10, 239-246, 1992.

Crossway, A., Oakes, J. V., Irvine, J. M., Ward, B., Knauf, V. C. and Shewmaker, C. K., "integration of foreign DNA following microinjection of tobacco mesophyll protoplasts", ***Mol. Gen. Genet***., 202, 179-185, 1986.

Dai, S., Zheng, P., Marmey, P., Zhang, S., Tian, W., Chen, S., Beachy, R.N. and Fauquet, C., "Comparative analysis of transgenic rice plants obtained by *Agrobacterium*-mediated transformation and particle bombardment", ***Molecular Breeding***, 7, 25-33, 2001.

DANIDA,"Assessment of Potentials and Constraints for Development and Use of Plant Biotechnology in Relation to Plant Breeding and Crop Production in Developing Countries",***Royal Danish Ministry of Foreign Affairs***, Copenhagen, Denmark, 2002. www.icsu.org/1_icsuscience/GMO/html/DANIDA%20Biblio%20Entry.htm

Datta, S., Peterhans, A., Datta, K. and Potrykus, I., "Genetically Engineered Fertile Indica-Rice Recovered From Protoplasts", ***Bio/*Technology**,8, 736-740, 1990.

Delbreil, B., Guerche, P. and Jullien, M., "*Agrobacterium*-mediated transformation of *Asparagus officinalis* L. long term embryogenic callus and regeneration of transgenic plants", ***Plant cell reports***, 12, 129-132, 1993.

Dhir, S.K., Pajeau, M.E., Frommn, M.E. and Fry, J.E., "Anthocyanin genes as visual markers for wheat transformation. Improvement of cereal quality by genetic engineering", ***Plenum***, 17, 737–741, 1994.

Dommisse, E., Development of an *Agrobacterium* transformation system for onion (*Allium cepa* L.), PhD thesis, ***Lincoln University***, New Zealand, 1-6, 1993.

Dommisse, E.M., Leung D.W.M., Shaw M.L. and Conner A.J., "Onion is a monocotyledonous host for *Agrobacterium*", ***Plant Science,*** 69, 249–257, 1990.

Dunstan, D.I. and Short, K.C., "Improved growth of tissue cultures of the onion, *Allium cepa*", ***Physiol. Plant***, 41, 70–72, 1977.

Eady, C.C., Kamoi, T., Kato, M., Porter, N.G., Davis, S., Shaw, M., Kamoi, A. and Imai, S., "Silencing onion lachrymatory factor synthase causes a significant change in the sulfur secondary metabolite profile", ***Plant Physiol***, 147, 2096–2106, 2008.

Eady, C.C., Davis, S., Farrant, J., Reader, J. and Kenel, F., "*Agrobacterium tumefaciens*-mediated transformation and regeneration of herbicide resistant onion (*Allium cepa*) plants", ***Annals of Applied Biology***, 142, 213–217, 2003.

Eady, C.C., Weld, R.J. and Lister, C.E., "*Agrobacterium tumefaciens*-mediated transformation and transgenic-plant regeneration of onion (*Allium cepa* L.)", ***Plant Cell Reports***, 19(4), 376-381, 2000.

Eady, C.C., Butler, R.C. and Suo, Y., "Somatic embryogenesis and plant regeneration from immature embryo cultures of onion (*Allium cepa* L.)", ***Plant Cell Rep***, 18, 111–116, 1998a.

Eady, C.C. and Lister, C.E., "A comparison of four selective agents use with *Allium cepa* L. immature embryos and immature embryo-derived cultures", ***Plant Cell Rep***, 18, 117–121, 1998b.

Eady, C.C., Lister, C.E., Suo, Y., Schaper, D. and Suo, Y.Y., "Transient expression of *uidA* constructs in in vitro onion (*Allium cepa* L.) cultures following particle bombardment and *Agrobacterium*-mediated DNA delivery", ***Plant Cell Rep***, 15, 958–962, 1996.

Eady, C.C., "Towards the transformation of onions (*Allium cepa*)", New Zealand, ***Journal of crop and horticultural science***, 23(3), 239-250, 1995.

Eady, C., Lindsey, K. and Twell, D., "The significance of microspore division and division symmetry for vegetative cell-specific transcription and generative cell differentiation", ***Plant cell***, 7(1), 65-74, 1995a.

Eady, C.; Lister, C. E.; Schafer, D. S., "An onion (*Allium cepa* L.) in vitro culture system developed for particle-gun mediated transformation", ***11th Biennial Conference of the New Zealand Branch of the International Association for Plant Tissue Culture***, Abstract, 1995b.

Engelen, F., Molthoff, J., Roosien, J. and Schouten, S., "Plantibodies: a new way to obtain disease resistant plants", ***4th International Congress of Plant Molecular Biology***, Amsterdam. Abstract-1914, 1994.

Elomaa, P., Honkanen, J., Puska, R., Seppanen, P., Helariutta, Y., Mehto, M., Kotilainen, M., Nevalainen, L. and Teeri, T., "*Agrobacterium* mediated transfer of antisense chalcone synthasesDNA to Gerbera hybrids inhibits flower pigmentation", ***Bio/Technology***, 11, 508-511, 1993.

FAO, "Major Food and Agricultural Commodities and Producers - Countries by Commodity", ***Food and Agriculture Organization of the United Nations***, 2014. www.Fao.org. Retrieved 2014-05-18.

Fennell, A. and Hauptmann, R., "Electroporation and PEG delivery of DNA into maize microspores", ***Plant cell reports***, 11, 567-570, 1992.

Finer, J.J. and Mcmullen, M.D., "Transformation of soybean via particle bombardment of embryogenic suspension culture tissue", ***In Vitro cellular and developmental Biology-Plant***, 27, 115-182, 1991.

Fraley, P.T., Rogers, S.G. and Horsch, R.B., "Genetic transformation in higher plants", CRC Crit. Rev. ***Plant Science***, 4, 1-46, 1986.

Fraley, R.T., Rogers, S.G., Horsch, R.B., Sanders, P.R., Flick, J.S., Adams, S.P., Bittner, M.L., Brand, L.A., Fink, C.L., Fry, J.S., Galluppi, G.R., Goldberg, S.B., Hoffmann, N.L. and Woo, S.C., "Expression of bacterial genes in plant cells", ***Proceedings of the National Academy of Sciences of the United States of America***, 80 (15), 4803–4807, 1983.

Frame, B.R., Drayton, P.R., Bagnall, S.V., Lewnau, C.J., Bullock, W.P., Wilson, H.M., Dunwell, J.M., Thompson, J.A. and Wang, K., "Production of fertile transgenic maize plants by silicon-carbide whisker-mediated transformation", ***Plant Journal,*** 6, 941-948, 1994.

Fromm, M.E., Taylor, L.P. and Walbot, V., "Stable transformation of maize after gene-transfer by electroporation", ***Nature***, 319, 791-793, 1986.

Gelvin, S.B., "*Agrobacterium*-mediated plant transformation: the biology behind the 'gene-jockeying' tool", ***Microbiol Mol Biol***, 67, 16–37, 2003.

GOP, "Minor Crops: Area, production and yield per hectare of onion. Economic Survey of Pakistan 2013-2014", ***Pakistan Bureau of Statistics (Economic Wing)***, Government of Pakistan, Islamabad, 2014.

Grant, D., "Onion cultivars grown overseas: characteristics and potential uses", ***New Zealand commercial grower***, 43 (1), 22, 1988.

Graves A.F., Goldman S.L., "The transformation of *Zea mays* seedlings with *Agrobacterium tumefaciens*", ***Plant Mol. Biol.***, 7, 43–50, 1986.

Hailekidan, B., Andargie, M. and Assefa, K.,"In Vitro Plantlet Regeneration from the Bulbs of Shallot (*Allium cepa* Var. Group *Aggregatum*)", ***Research in Plant Sciences***, 1(2), 45-52, 2013.

Hansen, G.; Wright, M., "Recent Advances in the transformation of plants", ***Trends In Plant Science***, 4, 226-231, 1999.

Hiei, Y., Ishida, Y. and Komari, T., "Progress of cereal transformation technology mediated by *Agrobacterium tumefaciens"*, ***Front Plant Sci***., 5, 628, 2014.

Hiei, Y., Komari, T. and Kubo, T., "Transformation of rice mediated by *Agrobacterium tumefaciens*", ***Plant Mol Biol***., 35(1), 205-218, 1997.

Hiei, Y., Ohta, S., Komari, T. and Kumashiro, T., "Efficient transformation of rice (*Oryza sativa* L.) mediated by *Agrobacterium* and sequence analysis of the boundaries of the T-DNA", ***Plant Journal***, 6, 271-282, 1994.

Hussey, G. and Falavigna, A., "Origin and production of in vitro adventitious shoots in the onion *Allium cepa* L.", ***Journal of experimental botany***, 31 (125), 1675-1686, 1980.

Jaynes, J.M., Nagpala, P., Destefano-Beltan, L., Huang, J.H., Kim, J.H., Denny, T., and Cetiner, S.,"Expression of a cecropin B lytic peptide analoguein transgenic tobacco confers enhanced resistanceto bacterial wilt caused by *Pseudomonas solanaceanim.* ***Plant science***, 89, 43-53, 1993.

Joubert, P., Sangwan, R.S., Aouad, M.E.A., Beaupere, D. and Norreel, B.S., "Influence of phenolic compounds on *Agrobacterium* vir gene induction and onion gene transfer", ***Phytochemistry,*** 40, 1623–1628, 1995.

Kaeppler, H., Somers, D., Rines, H. and Cockburn, A., "Silicon carbide fiber-mediated stabletransformation of plant cells", ***Theoretical and applied genetics***, 84, 560-566, 1992.

Kamata, Y., Masamura, N., Miyazaki, A. and Nagata, T., "A novel auto-fluorescence-based selection of calli amenable to *Agrobacterium*-mediated transformation in onion (*Allium cepa* L.)", ***Plant Biotechnology***, 28, 361–371, 2011.

Kamstaityte, D. and Stanys, V., “Micro-propagation of onion (*Allium cepa* L.)” ***Acta Universitatis Latviensis, Biology***, 676, 173–176, 2004.

Khanna, H.K. and Raina, S.K., "Genotype X Culture Media Interaction Effects on Regeneration Responseof 3 *Indica* Rice Cultivars", ***Plant cell, tissue and organ culture***, 52(3), 145-153, 1998.

Khar, A., Bhutani, R.D., Yadav, N. and Chowdhury, V.K., “Effect of explant and genotype on callus culture and Regeneration in onion (*Allium cepa*)”, ***Akdeniz Universitesi Ziraat Fakultesi Dergisi***, 18(3), 397-404, 2005.

Kramer, C., DiMaio, J., Carswell, G.K. and Shillito, R.D., “Selection of transformed protoplast derived *Zea mays* colonies with phosphinothricin and a novel assay using the pH indicator chlorophenol red”, ***Planta***, 190, 454-458, 1993.

Klein, T.M., Wolf, E., Wu, R. and Sanford, J., “High-velocity microprojectiles for delivering nucleic acids into living cells”, ***Nature***, 327, 70–73, 1987.

Kondo, T., Hasegawa, H. and Suzuki, M., "Transformation and regeneration of garlic (*Allium sativum* L.) by *Agrobacterium*-mediated gene transfer:, ***Plant Cell Reports***, 19, 989-993, 2000.

Logemann, J., Tigelaar, H., Sela-Buurlage, M.B., Ponstein, A.S., van Roekel, J.S.C., Bres-Vloemans, S.A., Dekker, I., Cornelissen, B.J.C., van den Elzen, P.J.M., Jongedijk, E. and Melchers, L.S., “Synergistic activity of chitinases and B -1-3-glucanase enhances Fusarum resistance in transgenic tomato plants”, ***4th International Congress of Plant Molecular Biology, Amsterdam***, Abstract-1901, 1994.

Lydiate, D., Dale, P., Lagercrantz, U., Parkin, Y. and Howell, P., “Selecting the optimum genetic background for transgenic varieties, with examples from brassica”, ***Euphytica***, 85, 351-358, 1995.

Malla, A., Srinivasan, B., Shanmugaraj, B.M. and Ramalingam, S., "Micropropagation and DNA delivery studies in onion cultivars of Bellary, CO3." ***J. Crop Sci. Biotechnol***., 18, 37, 2015.

Marinangeli, P.A., Zappacosta, D.C., Curvetto, N.R. and Galmarini, C.R., "Callus Induction and Plant Regeneration in Onion (*Allium cepa* L.)", ***Acta Hortic***., 688, 301-308, 2005.

Murashige, T. and Skoog, F., "A Revised Medium for Rapid Growth and Bio Assays with Tobacco Tissue Cultures", ***Physiologia Plantarum***, 15, 473–497, 1962.

Murray, M.G. and Thompson, W.F., "Rapid isolation of high molecular weight plant DNA", ***Nucleic Acids Res***., 8 (19), 4321–4326, 1980.

Nitsch, J. and Nitsch, C., "Haploid plants from pollen grains", ***Science***, 163, 85, 1969.

Ozawa. K., "A high-efficiency *Agrobacterium*-mediated transformation system of rice (*Oryza sativa* L.)", ***Methods Mol Biol.***, 847, 51-57, 2012.

Phillips, G. and Hubstenberger, J., "Plant regeneration in vitro of selected *Allium* species and interspecific hybrids", ***Hort. Science,*** 22(1), 124-125, 1987.

Phillips, G. and Luteyn, K., "Effects of picloram and other auxins on onion tissue cultures", ***Journal of the American Society for Horticultural Science***, 108(6), 948-953, 1983.

Pike, M. L., "Onion Breeding", In: Bassett, M.J. (ed.),"Breeding Vegetable Crops", ***AVI Publishing Company, Inc., New York***, 1, 357-394, 1986.

Pimental, D., Hunter, M.S., La-Gro, J.A., Efroymsoun, R.A., Landers, J.C., Mervis, F.T., McCarthy, C.A. and Boyal, A.E., "Benefits and risks of genetic engineering in agriculture", ***BioScience***, 39, 606-614, 1989.

Pitzschke, A. and Hirt, H., "New Insights into an Old Story: *Agrobacterium*-Induced tumor formation in plants by plant transformation", ***The EMBO Journal***, 29(6), 1021–1032, 2010.

Rabinowitch, H. and Brewster, J., "Onions and allied crops: Botany, physiology and genetics", ***CRC Press***,***Florida,***1, 113-134, 1990.

Ramakrishnan, M., Ceasar, S., Duraipandiyan, V., Daniel, M.A. and Ignacimuthu, S., "Efficacious somatic embryogenesis and fertile plant recovery from shoot apex explants of onion (*Allium cepa.* L.), ***In Vitro Cell Dev. Biol.—Plant***, 49 (3), 285-293, 2013.

Robinson, K. and Firoozabady, E., "Transformation of floriculture crops", ***Scientia horticulturae***, 55, 83-99, 1993.

Shahin, E. and Kaneko, K., "Somatic embryogenesis and plant regeneration from callus cultures of non-bulbing onions", ***Hort. Science***, 21(2), 294-295, 1986.

Sivanesan, I., Kyoung, Ke, Kyoung, Km., Young, Ke. and Park Sw., "Somatic embryogenesis and plant regeneration from zygotic embryo explants of onion", ***Horticultura Brasileira***, 33, 441-447, 2015.

Somers, D., Rines, H., Gu, W., Kaeppler, H. and Bushnell, W., "Fertile, transgenic oat plants", ***Bio/Technology***, 10, 1589-1594, 1992.

Thomas, J.C., Adams, D., Nessler, C., Bohnert, H.J. and Brown, J.K., "Reduced reproduction of whitefly (*Bemisia tabacii*) on transgenic tobacco expressing tryptophan decarboxylase", ***4th International Congress of Plant Molecular Biology***, Amsterdam, Abstract-1924, 1994.

Thompson, J.A., Frame, B.R., Drayton, P.R., Bagnall, S.V., Jiao, S., Lewnau, C.J., Bullock, W.P., Wilson, H.M., Dunwell, J.M. and Wang, K., "Production of fertile transgenic maize plants by silicon carbide fiber mediated transformation", ***4th International Congress of Plant Molecular Biology***, Amsterdam. Abstract-2062, 1994.

Valk, P., Scholten, O., Verstappen, F., Jansen, R. and Dons, J., "High frequency somatic embryogenesis and plant regeneration from zygotic embryo-derived callus cultures of three *Allium* species", ***Plant cell, tissue and organ culture***,30, 181-192. 1992.

Vasil, V., Srivastava, V., Castillo, A., Fromm, M. and Vasil, I., "Rapid production of transgenic wheat plants by direct bombardment of cultured immature embryos", ***Bio/Technology***, 11, 1553-1558, 1993.

Vasil, V., Castillo, A., Fromm, M. and Vasil, I., "Herbicide resistant fertile transgenic wheat plants obtained by microprojectile bombardment of regenerable embryogenic callus", ***Bio/Technology***, 10, 667-674, 1992.

Vaucheret, H., Beclin, C., Elmayan, T., Feuerbach, F., Godon, C., Morel, J.B., Mourrain, P., Palauqui, J.C., Vernhettes, S., "Transgene-induced gene silencing in plants", ***Plant Journal***, 16, 651-659, 1998.

Viterbo, A., Rabinowitch, H., Altman, A., "Plant regeneration from callus cultures of *Allium trifoliatum* subsp. *hirsutum* and assessment of genetic stability by isozyme polymorphism", ***Plant breeding***, 108,265-273, 1992.

Wan, Y., Lemaux, P.G., "Generation of large numbers of independently transformed fertile barley plants", ***Plant physiology***, 104, 37-48, 1994.

Wang, Z., Takamizo, T., Iglesais, V., Osusky, M., Nagel, N., Potrykus, I. and Spangenberg, G., "Transgenic plants of tall fescue (*Festuca arundinacea*) obtained by direct gene transfer to protoplasts", ***Bio/Technology***, 10, 691-694, 1992.

Weeks, J., Anderson, O. and Blechl, A., "Rapid production of multiple independent lines of fertile transgenic wheat (*Triticum aestivum*)", ***Plant physiology***, 102, 1077-1084, 1993.

Wilmink, A. and Dons, J., "Selective agents and marker genes for use in transformation of monocotyledonous plants", ***Plant molecular biology reporter***, l1 (2), 165-185, 1993.

Wu, X., Yang, F., Piao, X.C., Li, K.H., Lian, M.L. and Dai, Y., "High-frequency plantlet regeneration by somatic embryogenesis from mature zygotic embryos of onion", ***New Zealand Journal of Crop and Horticultural Science***, 43 (4), 249-260, 2015.

Xu, Q.G. and Cui, C.R., "Genetic transformation of OSISAP1 gene to onion (*Allium cepa* L.) mediated by micro projectile bombardment", ***Journal of Plant Physiology and Molecular Biology***, 33 (3), 188-96, 2007.

Xu, X. and Li, B., "Fertile transgenic *Indica* rice plants obtained by electroporation of the seed embryo cells", ***Plant cell reports***, 13, 237-242, 1994.

Zheng, S.J., Henken, B., de Maagd, R.A., Purwito, A., Krens, F.A. and Kik, C., "Two different *Bacillus thuringiensis* toxin genes confer resistance to beet armyworm (*Spodoptera exigua Hübner*) in transgenic Bt-shallots (*Allium cepa* L.). ***Trans Res***., 14, 261–272, 2005.

Zheng, S., Khrustaleva, L., Henken, B., Sofiari, E., Jacobsen, E., Kik, C. and Krens, F.A., "*Agrobacterium tumefaciens*-mediated transformation of *Allium cepa* L.: the production of transgenic onions and shallots", ***Molecular Breeding***, 7 (2), 101-115, 2001.

Zheng, S.J., Henken, B., Sofiari, E., Keizer, P., Jacobsen, E., Kik, C. and Krens, F.A., "Effect of cytokinins and lines on plant regeneration from long-term callus and suspension cultures of *Allium cepa* L.", ***Euphytica***, 108 (2), 83-90, 1999.

Zheng, S., Henken, B., Sofiari, E., Jacobsen, E., Krens, F.A. and Kik, C., "Factors influencing induction, propagation and regeneration of mature zygotic embryo derive callus from *Allium cepa*", ***Plant cell, tissue and organ culture***, 53 (2), 99-105, 1998.

APPENDICES

Appendix-A Average In-vitro Germination (%age) of Onion Cultivars

Cultivars	Day Response	Average Germination %
Sec	Intermediate	78
Sampiyon	Intermediate	93
Burgaz	Intermediate	77
Hazar	short	15
15001	Short	45
White grand	Short	10
Taraz	Long	75
Kral	Long	78
Bereket	Long	13

Appendix-B PCR Screening of primary transformants

Plant #	Number assigned to sample	PCR result
1	501	-
2	502	+
3	503	-
4	504	-
5	505	-
6	506	-
7	507	-
8	508	-
9	509	-
10	510	-
11	511	-
12	512	-
13	513	-
14	514	-
15	515	-
16	516	-
17	517	-
18	518	-
19	519	-
20	520	-
21	521	-
22	522	+
23	523	-
24	524	-
25	525	+
26	526	-
27	527	+
28	528	-
29	529	+
30	530	-
31	531	-
32	532	-
33	533	-
34	534	-

Appendix-B (Continue) PCR Screening of primary transformants

Plant #	Number assigned to sample	PCR result
35	535	-
36	536	-
37	537	-
38	538	-
39	539	-
40	540	-
41	541	+
42	542	-
43	543	-
44	544	-
45	545	-
46	546	-
47	547	-
48	548	-
49	549	-
50	550	+
51	551	-
52	552	+
53	553	-
54	554	+
55	555	-
56	556	-
57	557	-
58	558	-
59	559	+
60	560	+
61	561	-
62	562	-
63	563	-
64	564	-
65	565	-
66	566	-
67	567	-
68	568	-

Appendix-B (Continue) PCR Screening of primary transformants

Plant #	Number assigned to sample	PCR result
69	569	-
70	570	-
71	571	-
72	572	-
73	573	-
74	574	-
75	575	-
76	576	+
77	577	+
78	578	-
79	579	+
80	580	+
81	581	+
82	582	+
83	583	+
84	584	+
85	585	-
86	586	+
87	587	-
88	588	-
89	589	-
90	590	+
91	591	-
92	592	+
93	593	+
94	594	+
95	595	+
96	596	+
97	597	+
98	598	+
99	599	-
100	600	+
101	601	+
102	602	+

Appendix-B (Continue) PCR Screening of primary transformants

Plant #	Number assigned to sample	PCR result
103	603	+
104	604	+
105	605	+
106	606	+
107	607	+
108	608	+
109	609	-
110	610	-
111	611	-
112	612	+
113	613	-
114	614	-
115	615	+
116	616	+
117	617	-
118	618	+
119	619	+
120	620	+
121	621	+
122	622	+
123	623	+
124	624	-
125	625	-
126	626	-
127	627	+
128	628	-
129	629	-
130	630	-
131	631	+
132	632	+
133	633	-
134	634	-
135	635	-
136	636	-

Appendix-B (Continue) PCR Screening of primary transformants

Plant #	Number assigned to sample	PCR result
137	637	+
138	638	+
139	639	-
140	640	-
141	641	-
142	642	-
143	643	-
144	644	-
145	645	-
146	646	-
147	647	-
148	648	-
149	649	-
150	650	-
151	651	-
152	652	-
153	653	-
154	654	-
155	655	-
156	656	+
157	657	-
158	658	-
159	659	+
160	660	-
161	661	-
162	662	-
163	663	+
164	664	+
165	665	-
166	666	-
167	667	-
168	668	-
169	669	-
170	670	-

Appendix-B (Continue) PCR Screening of primary transformants

Plant #	Number assigned to sample	PCR result
171	671	-
172	672	-
173	673	-
174	674	-
175	675	-
176	676	+
177	677	-
178	678	-
179	679	-
180	680	+
181	681	-
182	682	+
183	683	+
184	684	-
185	685	+
186	686	-
187	687	-
188	688	+
189	689	+
190	690	-
191	691	-
192	692	-
193	693	-
194	694	-
195	695	-
196	696	-
197	697	-
198	698	-
199	699	-
200	700	-
201	701	-
202	702	-
203	703	-
204	704	-

Appendix-B (Continue) PCR Screening of primary transformants

Plant #	Number assigned to sample	PCR result
205	705	-
206	706	-
207	707	+
208	708	-
209	709	+
210	710	-
211	711	-
212	712	-
213	713	-
214	714	-
215	715	-
216	716	+
217	717	-
218	718	-
219	719	-
220	720	+
221	721	+
222	722	+
223	723	+
224	724	-
225	725	-
226	726	+
227	727	-
228	728	-
229	729	-
230	730	+
231	731	-
232	732	-
233	733	+
234	734	-
235	735	-
236	736	-
237	737	-
238	738	-

Appendix-B (Continue) PCR Screening of primary transformants

Plant #	Number assigned to sample	PCR result
239	739	-
240	740	-
241	741	-
242	742	-
243	743	+
244	744	-
245	745	-
246	746	+
247	747	-
248	748	-
249	749	-
250	750	-
251	751	-
252	752	-
253	753	+
254	754	+
255	755	+
256	756	+
257	757	-
258	758	-
259	759	-
260	760	-
261	761	-
262	762	-
263	763	-
264	764	-
265	765	-
266	766	+
267	767	+
268	768	+
269	769	-
270	770	-
271	771	+
272	772	-

Appendix-B (Continue) PCR Screening of primary transformants

Plant #	Number assigned to sample	PCR result
273	773	-
274	774	-
275	775	-
276	776	-
277	777	-
278	778	-
279	779	+
280	780	-
281	781	+
282	782	+
283	783	-
284	784	-
285	785	-
286	786	-
287	787	-
288	788	-
289	789	-
290	790	-
291	791	-
292	792	-
293	793	-
294	794	-
295	795	-
296	796	-
297	797	-
298	798	-
299	799	-
300	800	-
301	801	-
302	802	-
303	803	-
304	804	-
305	805	+
306	806	-

Appendix-B (Continue) PCR Screening of primary transformants

Plant #	Number assigned to sample	PCR result
307	807	-
308	808	-
309	809	-
310	810	-
311	811	-
312	812	-
313	813	-
314	814	-
315	815	-
316	816	-
317	817	-
318	818	-
319	819	-
320	820	-
321	821	-
322	822	-
323	823	-
324	824	-
325	825	-
326	826	-
327	827	-
328	828	-
329	829	-
330	830	-
331	831	-
332	832	-
333	833	-
334	834	-
335	835	-
336	836	-
337	837	-
338	838	-
339	839	-
340	840	-

Appendix-B (Continue) PCR Screening of primary transformants

Plant #	Number assigned to sample	PCR result
341	841	-
342	842	-
343	843	-
344	844	-
345	845	-
346	846	-
347	847	-
348	848	-
349	849	-
350	850	-
351	851	-
352	852	-
353	853	-
354	854	+
355	855	-

* From Sample # 501-583RSM-1 (Sampiyon)

* From Sample # 584-647 RSM-1 (Kral)

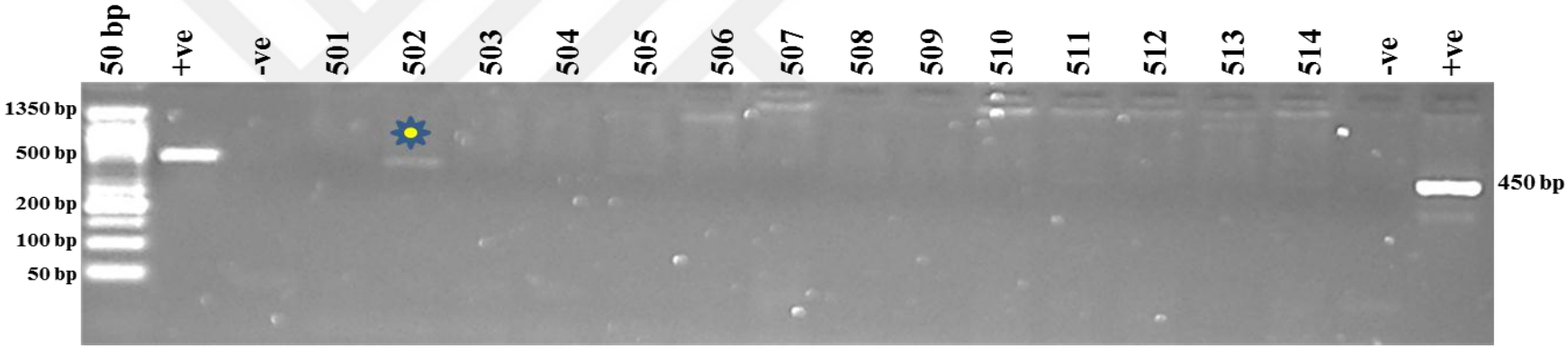
*From Sample # 648-718.RSM-2 (Sampiyon)

* From Sample # 719-776 RSM-2 (Kral)

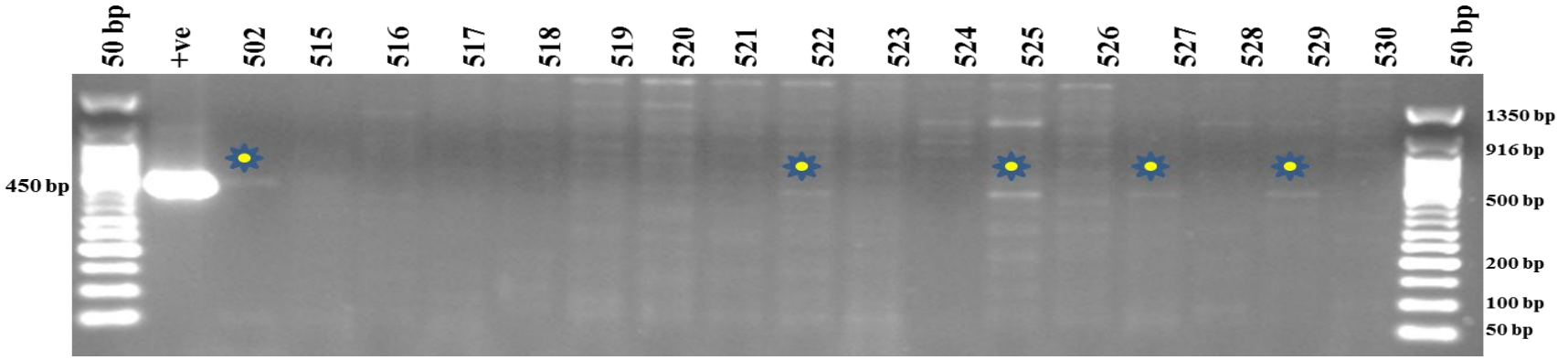
* From Sample # 777-808RSM-3 (Sampiyon)

* From Sample # 809-855 RSM-3 (Kral)

Appendix-C Screening of Transgenic Plants through PCR

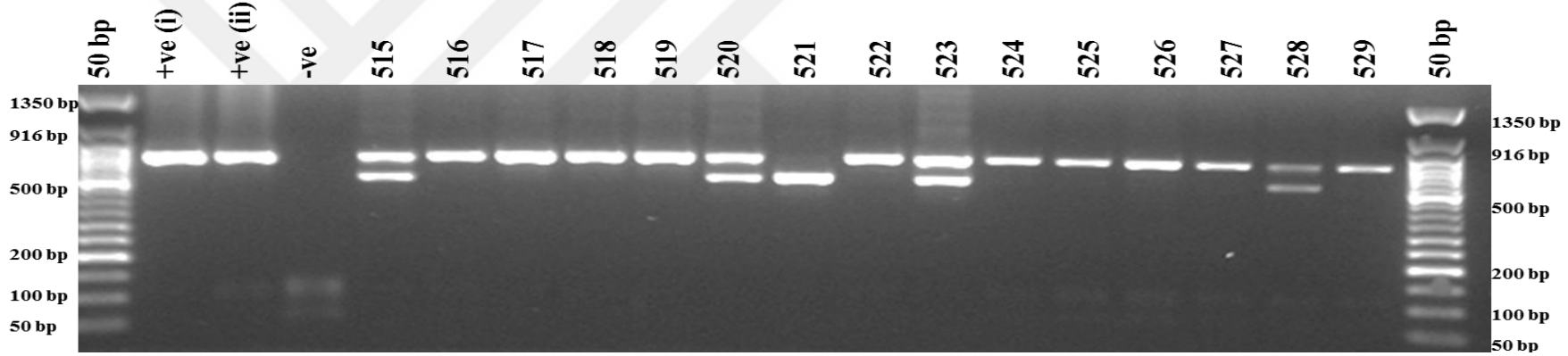


Appendix-C PCR screening for the presence of 450 bp fragment of *nptII* gene, 50bp DNA Ladder; **+ve=** positive control (PRD-400 Plasmid); **-ve**= without DNA control; Lane 501-514 putative plants

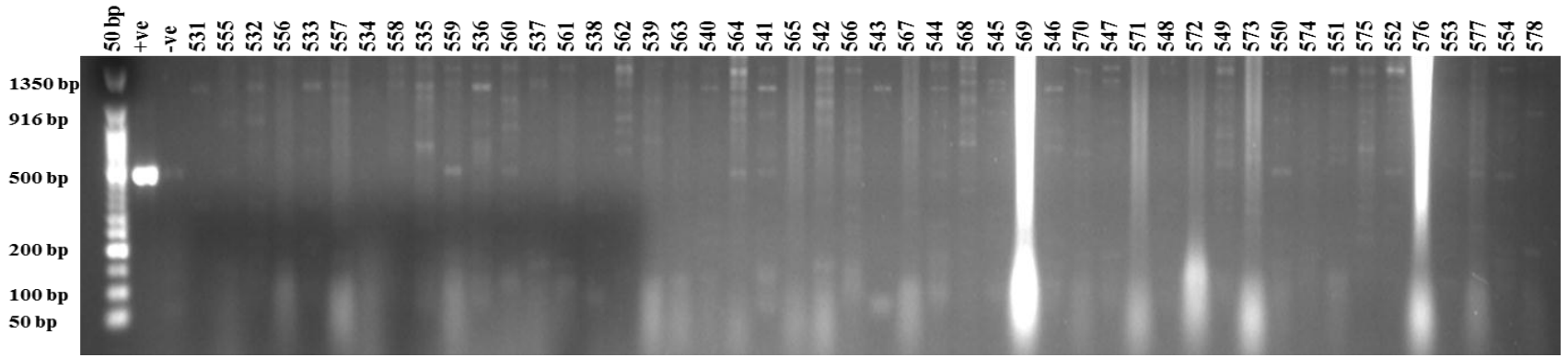


Appendix-C PCR screen for the presence of 450 bp fragment of *nptII* gene, 50bp DNA Ladder; **+ve=** Positive control (PRD-400 Plasmid); **-ve**= without DNA control; Lane 502-530 putative plants

Appendix-C (Continue) Screening of Transgenic Plants through PCR

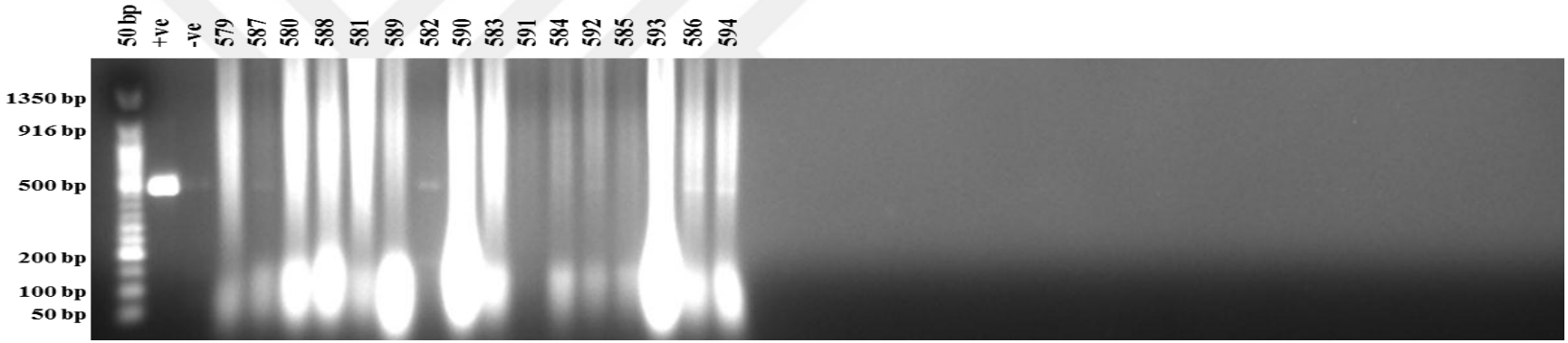


Appendix-C Checking of DNA quality with GAF primers (GAF-08, GAF- 09), 50bp DNA Ladder; +**ve (i) =** Positive control (B17-50A), +ve (ii) = Positive control (B17-50B); **-ve**= without DNA control.

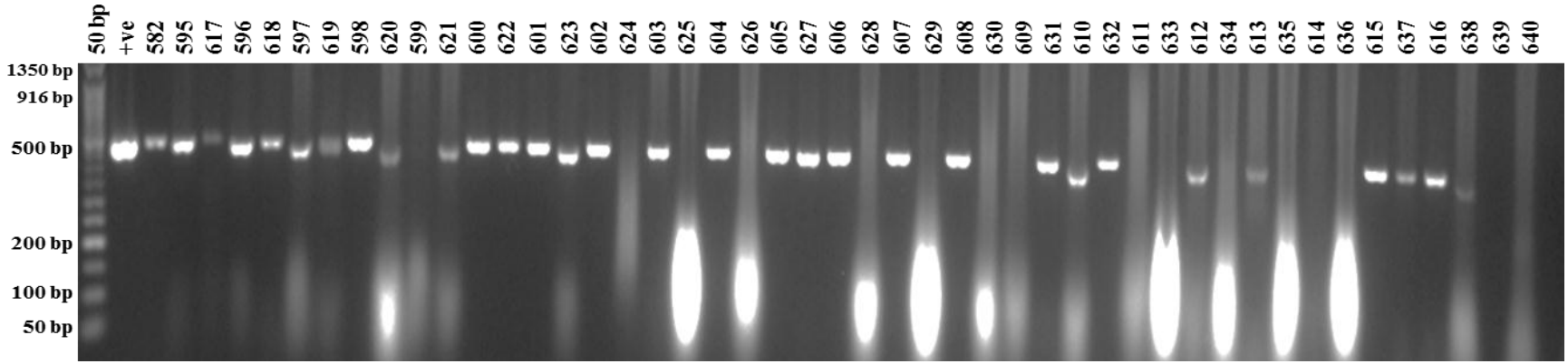


Appendix-C PCR screening for the presence of 450 bp fragment *nptII* gene, 50bp DNA Ladder; **+ve=** Positive control (PRD-400 Plasmid); **-ve**= without DNA control; Lane 531-578 putative plants

Appendix-C (Continue) Screening of Transgenic Plants through PCR

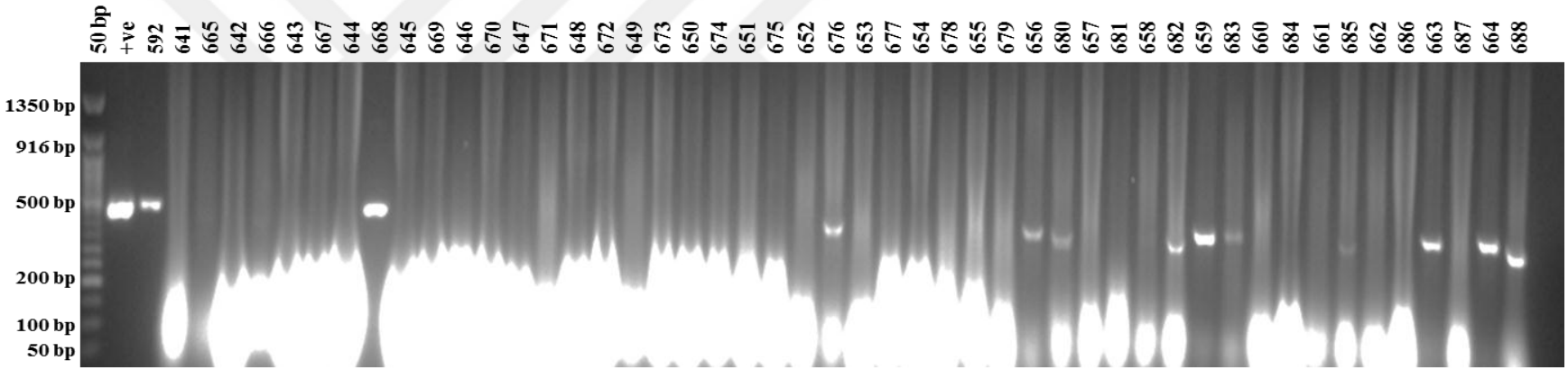


Appendix-C PCR screening for the presence of 450 bp fragment of *nptII* gene, 50bp DNA Ladder; **+ve=** Positive control (PRD-400 Plasmid); **-ve**= without DNA control; Lane 579-594 putative plants

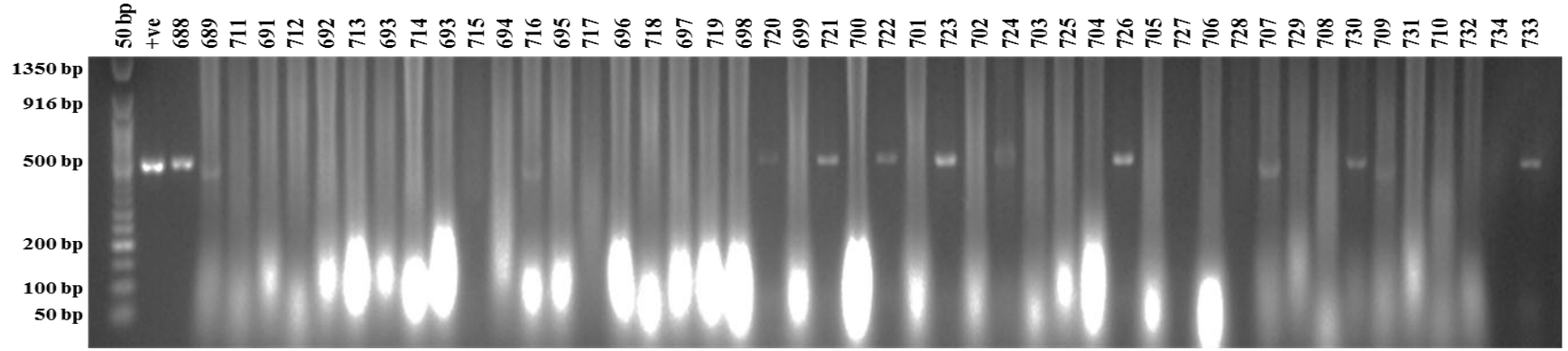


Appendix-C PCR screening for the presence of 450 bp fragment of *nptII* gene, 50bp DNA Ladder; **+ve=** Positive control (PRD-400 Plasmid); **-ve**= without DNA control; Lane 582-640 putative plants

Appendix-C (Continue) Screening of Transgenic Plants through PCR

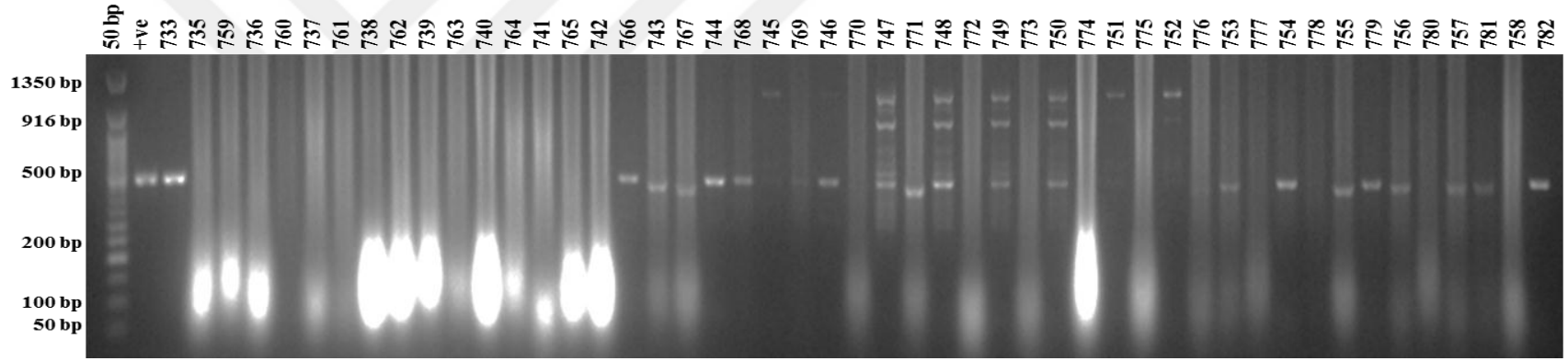


Appendix-C PCR screening for the presence of 450 bp fragment of *nptII* gene, 50bp DNA Ladder; **+ve=** Positive control (PRD-400 Plasmid); **-ve**= without DNA control; Lane 592-688 putative plants

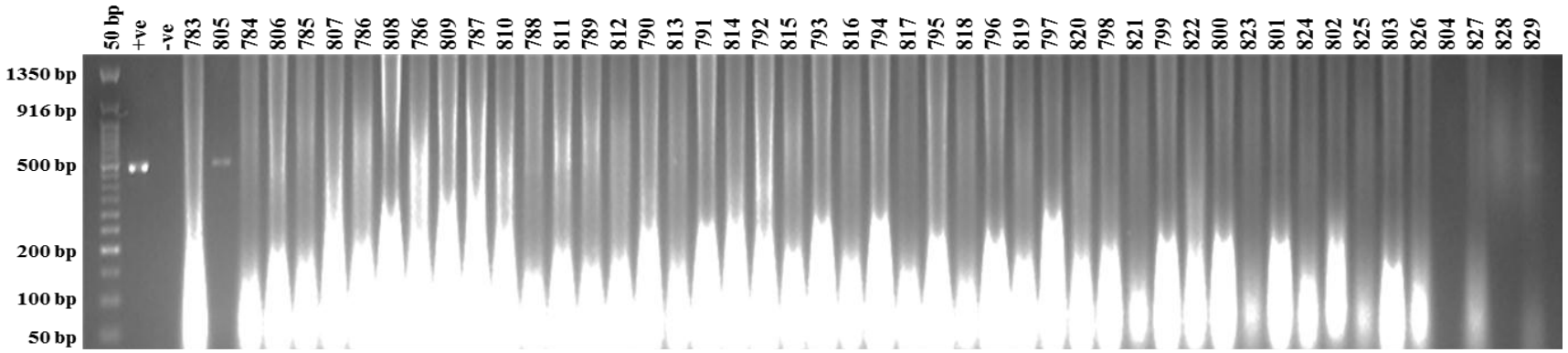


Appendix-C PCR screening for the presence of 450 bp fragment of *nptII* gene, 50bp DNA Ladder; **+ve=** Positive control (PRD-400 Plasmid); **-ve**= without DNA control; Lane 688-733 putative plants

Appendix-C (Continue) Screening of Transgenic Plants through PCR

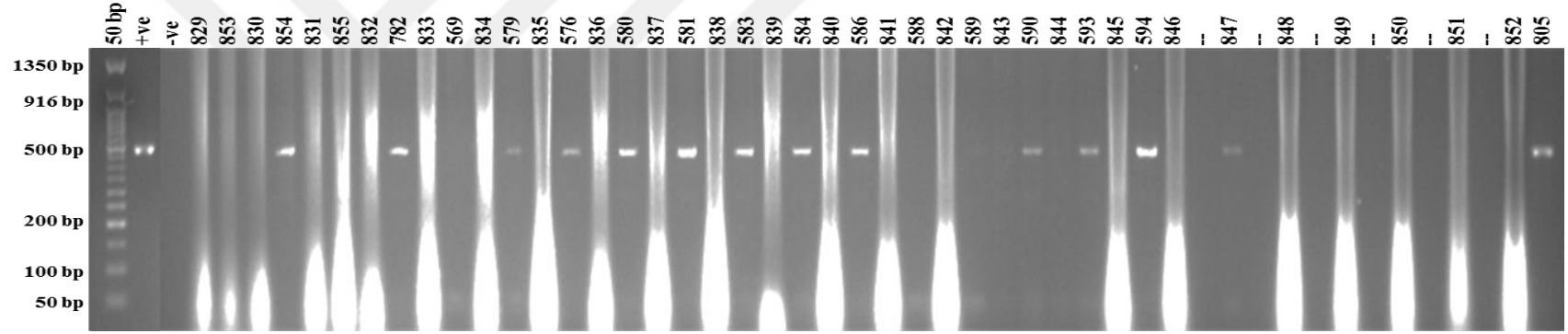


Appendix-C PCR screening for the presence of 450 bp fragment of *nptII* gene, 50bp DNA Ladder; **+ve=** Positive control (PRD-400 Plasmid); **-ve**= without DNA control; Lane 733-782 putative plants



Appendix-C PCR screening for the presence of 450 bp fragment of *nptII* gene, 50bp DNA Ladder; **+ve=** Positive control (PRD-400 Plasmid); **-ve**= without DNA control; Lane 783-829 putative plants

Appendix-C (Continue) Screening of Transgenic Plants through PCR



Appendix-C PCR screening for the presence of 450 bp fragment of *nptII* gene, 50bp DNA Ladder; **+ve=** Positive control (PRD-400 Plasmid); **-ve**= without DNA control; Lane 829-805 putative plants

CURRICULUM VITAE

Khazina AMIN was born on December 24, 1992 in Faisalabad, Pakistan. She completed her higher secondary education from Faisalabad College for Women, Faisalabad in 2010. Afterwards she joined University of Agriculture, Faisalabad Pakistan in 2010 for her undergraduate studies. She completed her B.Sc. (Hons) in Agriculture majoring in Plant Breeding and Genetics in 2014. She won MS leading PhD fellowship (Graduate Scholarship Program for International Students-2215) awarded by The Scientific and Technological Research Council of Turkey (TUBITAK) and enrolled in Graduate School of Natural and Applied Sciences, Department of Agricultural Genetic Engineering, Ömer Halisdemir University, Nigde, Turkey for her master studies under the guidance of Dr. Ali Fuat GÖKÇE. During his master thesis research she worked on optimization of *Agrobacterium tumefaciens*-mediated transformation in onion (*Allium cepa* L.).