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REVIEW in POLISH BOTANY CENTENNIAL

Somatic Embryogenesis, Genetic Modification, and GMOs at the Department of Plant Biotechnology and Cytogenetics at the IHAR

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Abstract

At the Plant Breeding and Acclimatization Institute, National Research Institute (IHAR), research on plant tissue cultures began in 1970s. Some in vitro methods, such as anther cultures or isolated microspore cultures, have been directly used to produce homozygous lines in plant breeding. These methods are also a prerequisite for genetic manipulation for both scientific and practical purposes. Here, we describe the achievements of the Department of Biotechnology and Cytogenetics over the last few decades in the area of somatic embryogenesis of cereals, genetic modifications, GMO detection and identification, and GMO legislation. This long-term research on plant regeneration systems has led to the development of the first transgenic triticale plants in the world and the first transgenic plants in Poland, which was followed by a multi-generation study of triticale transgene stability. The gene flow of transgenic triticale and transgenic maize investigated in field studies under Polish environmental conditions, as well as the evaluation of triticale pollen flow, provided scientific data for the development of coexistence measures and GMO risk assessment in case of plant authorization for cultivation. Based on the experience gained over the years, a GMO Controlling Laboratory was established to support the official control of GMOs in Poland and the EU. It was later nominated as one of the National Reference Laboratories collaborating with the Joint Research Center (JRC) of the European Commission, providing scientific and technical support in areas related to health and consumer protection. The GMO Controlling Laboratory is accredited by the Polish Center for Accreditation according to the ISO 17025 standard, ensuring high-quality analyses and flexible accreditation scope. It participates in the ongoing debate in Poland and the EU on the legal aspects of new genomic techniques in plant breeding through publications, lectures at scientific conferences, and by providing expertise to relevant bodies.

Keywords

cereals; in vitro cultures; haploid embryos; genetic engineering; GMO control and legislation; reference laboratory for GMOs

1. Introduction

For thousands of years, people have been looking for ways to increase yield and improve food quality by breeding new crop varieties. The crops we use today are the result of selecting the most valuable genotypes and carefully analyzing the offspring. The introduction of new traits, such as disease resistance, or the creation of more efficient combinations of parental genes in the offspring to ensure better product quality, usually took more than 10 years. Currently, shortening the breeding process

is the goal of scientists and breeders. Therefore, breeders, in addition to classical procedures, apply new scientific methods to accelerate the breeding of the desired genotypes (J. Zimny & Michalski, 2019). The most recent method is the direct gene transfer method. It is characterized by high precision and provides detailed information on the traits of the expected final product (Sowa et al., 2021). Conventional breeding methods, in vitro culture, and genetic engineering are complementary methods for creating the desired varieties. Contemporary agriculture has different objectives, depending on its geographical location and economic conditions. In North America, it is the efficiency of production that counts; in Asia, it is the ability to meet the needs of the population; and in Europe, it is the challenge of sustainable agriculture. Owing to innovative technologies, breeding is becoming increasingly precise and fast to respond to the needs of changing markets on local and global scales. Over the past 35 years, genetic engineering, combined with tissue culture methods, has created the possibility of introducing DNA into the cells of various plant species and subsequently regenerating plants with new properties (Junker et al., 1987; Twardowski et al., 2003). Over the last 25 years, genetically modified (GM) crops obtained using recombinant DNA technology have been successfully cultivated by millions of farmers worldwide. The challenges for Polish breeders are competition from foreign companies, level of investment in research and development, and implementation of innovations in breeding. To meet these requirements, it is necessary to apply the latest scientific achievements, together with new breeding techniques. These include various variants of genetic transformation, but most importantly, editing of the genome through directed mutagenesis or modification. The development of new breeding techniques clashes with the problem of asynchronous authorization of the breeding products on a continental and global scale (Woźniak et al., 2020). However, science must develop independently from political turmoil.

The establishment of a very efficient plant regeneration system is the first prerequisite for any type of plant transformation experiment. Among various techniques, the electroporation of protoplasts and plant regeneration from GM protoplasts is the most difficult for dicots and even more for monocots. Before the first transformation could be done on cereal plants, it was necessary to develop in vitro plant regeneration protocol, which would enable the introduction of foreign genes into the cereal genome and the regeneration of plants with new genes and traits. Several varieties of cereal explants suitable for in vitro cultures have been studied by many authors. Even roots have been examined, which in the case of rice proved to be applicable (J. Zimny & Lörz, 1986), but not in the case of wheat (Chin & Scott, 1977; Dudits et al., 1975). In the late 1970s and early 1980s, it was demonstrated that immature embryos are capable of forming embryogenic callus at a fairly high frequency, and many plants can be derived from such calluses (Bajaj, 1986; Carman et al., 1987; He et al., 1986; Heyser et al., 1985; Maddock et al., 1983; Ohnoutkova et al., 1984; Ozias-Akins & Vasil, 1982; T. Shimada & Yamada, 1979). In Poland, cereals were generally considered to be difficult plants for in vitro cultures, and perhaps this was the reason why only a few research centers performed in vitro studies on rye (Rybczyński, 1978a, 1978b, 1980; Rybczyński & Zduńczyk, 1986) and barley (Grünewaldt & Malepszy, 1975; Rybczyński et al., 1986). At the end of the 1980s, in vitro research on wheat started at Plant Breeding and Acclimatization Institute, National Research Institute (IHAR) in Radzików and resulted in the development of a system allowing to induce the somatic embryos from different explants (Menke-Milczarek & Zimny, 1992a, 1992b). This system was used to select plants resistant to deoxynivalenol, a mycotoxin produced by pathogens of the genus *Fusarium* (Menke-Milczarek & Zimny, 1991). At the end of the 1990s, the wheat somatic embryogenesis system was used by Prof. Jan Rybczyński from Botanical Garden of the Polish Academy of Sciences in collaboration with the University of Nebraska (USA) to achieve high transformation efficiency and long-term embryogenic potential of transformed tissue (Zhang et al., 2000). As far as triticale is concerned, the first works on callus induction and plant regeneration were recorded in the mid of 1980s (Stolarz & Lörz, 1986; J. Zimny & Rybczyński, 1986). These works were carried out in the Botanical Garden of the Polish Academy of Sciences, Powsin and IHAR, Radzików in cooperation with Dr. Horst Lörz from the Max Planck Institute in Cologne.

Research on plant tissue cultures and their uses in genetic engineering aimed to enrich the possibilities of modern plant breeding has been conducted at the IHAR since the 1970s. Here, we describe in detail the achievements of the Department of Biotechnology and Cytogenetics, a special unit of the IHAR, in the field of cereal somatic embryogenesis, genetic modification, GMO detection and identification, and GMO legislation.

2. Somatic Embryogenesis as a Prerequisite for Genetic Modification

In the 1980s, obtaining somatic embryos from monocots, especially cereals, was a major challenge for researchers worldwide (J. Zimny & Michalski, 2019). Scientists from IHAR, in collaboration with other Polish and German institutions, conducted pioneering studies that led to the description of somatic embryogenesis in triticale and rye. In 1985, an article on somatic embryogenesis in rye appeared at the Eucarpia meeting (Rybczyński & Zimny, 1985). One year later, the world's first reports on somatic embryogenesis in triticale were published (Stolarz & Lörz, 1986; J. Zimny & Rybczyński, 1986). These studies identified and described the structures emerging from calluses as somatic embryos. Histological analysis performed with electron microscopy revealed that these somatic embryos had all the characteristics of the zygotic embryos (J. Zimny & Lörz, 1989). Both induction of somatic embryogenesis and efficient plant regeneration had to be optimized. This was performed in the late 1980s by developing appropriate protocols. In subsequent years, we focused on triticale as a cereal of growing economic importance in Poland. Embryogenic lines derived from single plants of different genotypes were selected during several growing seasons. The best results were obtained with the MAH 1590 winter triticale line, in which high somatic embryogenesis efficiencies were recorded for all plants tested in subsequent experiments. Some lines from the other two genotypes showed a trend of decreasing efficiency in the following years. The question of what caused the observed variation within the stabilized genotypes must be answered. One possible explanation is the lack of genetic stability in evolutionarily young species such as triticale. Therefore, we developed a method to obtain homozygous lines by inducing haploid embryos from microspores. Statistical analysis showed no significant variation in somatic embryogenesis potential among MAH 1590 individuals with a very high level of somatic embryogenesis efficiency (>83%). At the same time, significant variability was observed among plants of the other genotypes tested. The MAH 1590 line, showing the highest embryogenesis efficiency, was at the same time the most uniform in terms of in vitro responsiveness. Therefore, this genotype was used in subsequent studies on the genetic modification of triticale. In a short time, MAH 1590 was registered by Dr. Bogusław Łapiński as 'Bogo,' a new high-yielding winter triticale variety. 'Bogo' has been successfully grown for many years in Poland, and after 30 years since our discovery, it continues to serve as a model genotype used in studies on in vitro plant regeneration and transformation of triticale (Hensel et al., 2012; Michalski et al., 2021; Oleszczuk et al., 2004; Sowa et al., 2005).

3. Genetic Modification of Cereals

Genetic engineering is currently used in both research and practice (Czaplicki et al., 2005; J. Zimny & de Vries, 2005). At the beginning of the new millennium, it was difficult to predict; however, the introduction of GM plants into the environment would face strong opposition from European society (Bock & Zimny, 2005; Twardowski et al., 2003). In our laboratories, the first studies on plant transformation were carried out using triticale plant material developed at IHAR, but our methods were later used for the transformation of cereal plants in other laboratories. The list of transgenic plants is long, but their reports are often vague and misleading. According to Potrykus (1991), proving an integrative transformation requires meeting several conditions, including complete Southern Blot analysis (signal in the region of high molecular weight), the presence of a complete gene, the absence of DNA contamination (negative control), and data allowing to distinguish false and true transformants. Fulfillment of these requirements by the transgenic triticale plants developed in IHAR was a result of

cooperation with leading European laboratories in 1993. These triticale plants contained a gene conferring resistance to the Basta herbicide. This was the world's first transgenic triticale (J. Zimny et al., 1995). The Polish Academy of Sciences awarded this achievement in 1996. In the following year, other cereals, such as rye, were also successfully transformed (Sowa et al., 2000).

4. Multi-Generation Analysis of Transgenic Triticale Plants

Transgenic lines have been stabilized by the regeneration of homozygous androgenic lines (Oleszczuk & Lukaszewski, 2014). Several generations of transgenic plants have been tested over the past 29 years. The most interesting finding was the localization of transgenes on individual chromosomes, which indicated the completely random nature of the insertion of the foreign gene into the recipient genome (Pedersen et al., 1997). This was the starting point for many studies, not only on genetic transformation but also on the potential negative impact of transgenic cereal plants on the environment. These studies were carried out in various Polish laboratories after 2000, and among many others, included a project on feeding successive generations of experimental mice with transgenic triticale (Baranowski et al., 2006; Jaszczak et al., 2008; Krzyżowska et al., 2010). The latter was conducted by IHAR in cooperation with the Institute of Genetics and Animal Biotechnology, Polish Academy of Sciences, Jastrzębiec and with the Faculty of Veterinary Medicine, Warsaw University of Life Sciences – SGGW.

5. Legal Situation and Risk Assessment of Transgenic Plants

After the first transgenic plants appeared in the United States in the 1990s, an intense debate began on the potential negative impact of all GMOs, especially GM plants (J. Zimny & de Vries, 2005). Polish law on GMOs came into force in 2001, and we were involved in preparing the legislation in the form of workshops and publications under the project GF/1200-98-84 financed by the United Nations Environment Program (Anioł et al., 1998, 1999a, 1999b, 1999c; Zimnoch-Guzowska et al., 2004). In 2006, the cultivation of GM maize MON 810, resistant to European corn borers, was introduced in Poland. In 2007, it was grown on 300 ha, and a year later, on approximately 3,000 ha. However, placing GM seeds in the market was later banned in Poland, so farmers had to buy seeds from other countries of the European Union (EU). MON 810 maize was rapidly gaining popularity, while at the same time raising voices claimed that these new biotechnological products had never been tested under Polish conditions. In response to this outcry, we began preparations for a special grant proposal because we already had some experience in this area resulting from similar projects, conducted together with Prof. Andrzej Anioł.

- 2000–2005: Genetically Modified Plant Network – grant – 5 Framework of European Commission.
- 2001–2005: PHARE PL/2001/IB/EN/03 "Implementation of biosafety systems in Poland."
- 2002–2005: UNEP/GEF "Support for the implementation of the national biosafety framework for Poland" contract No: GFL/2716-02-4531 (PMS:3010-02-05).
- 2004–2007: European Commission Project, Transition Facility 2004/016-829.03.01. "Strengthening environmental information in particular in the field of biosafety."

The grant proposal was successful, and in the years 2008–2011, after receiving funds from the National Centre for Research and Development, we carried out the research project (No. PBZ-MNiSW-06/1/2007) titled "Environmental and economic aspects of the authorization of the cultivation of genetically modified plants in Poland."

6. Gene Flow and the Coexistence of Genetically Modified, Conventional, and Organic Farming Systems

6.1. Case Study – Maize

The coexistence of conventional and GM maize (MON 810) was investigated in a 2-year study. MON 810 maize is the first and only transgenic crop authorized for cultivation in the EU. It was developed by introducing the *cry1ab* gene from the soil bacterium *Bacillus thuringiensis* into the maize genome. This gene is responsible for the production of Cry1Ab protein, which reduces the feeding of larvae of the most important maize pest, the European corn borer (*Ostrinia nubilalis*). This protein is considered harmless to other insects and mammals, including humans (Romeis et al., 2008; N. Shimada et al., 2003).

The level of outcrossing between conventional and transgenic maize varieties was examined to determine their coexistence principles under Polish farming conditions. Field studies were conducted during both seasons. Grain samples collected in the field were analyzed by quantitative real-time PCR. An isolation distance of 20 m was sufficient to assure the 0.9% labeling threshold for food and feed required by Regulation (EC) 1829/2003 (Grelewska et al., 2011).

6.2. Case Study – Triticale

Triticale is a self-pollinating crop. However, low levels of outcrossing are usually observed, depending on the environmental conditions. Our project was designed to establish the minimum isolation distance for GM, conventional, and organic triticale cropping systems. Coexistence, in the case of triticale, depends on climatic and biological factors, as well as agronomic practices. The most important factor is the possible gene flow mediated by wind-borne pollen and potential cross-fertilization with GM triticale growing in neighboring fields. Our field experiments were conducted using transgenic lines expressing *uid*A (J. Zimny et al., 1995) and the isogenic triticale variety 'Bogo.' We used a homozygous triticale line originally transformed with plasmid pDB1. Passive traps for pollen monitoring were spaced at increasing distances from transgenic triticale. Pollen concentrations vary considerably depending on distance, wind direction, air temperature, and year of monitoring. We observed a largely consistent positive effect of air warming on the number and spread of pollen grains at a distance of 85 m. The pollen density near the pollinator was very high and only several pollen grains could be detected up to 85 m. Gene flow was studied in two experimental variants, "spatial isolation" where conventional triticale was planted at various distances from the GM triticale pollinator field, and "border rows" where GM triticale pollinator field was surrounded by conventional triticale plants. Based on the results of cross-pollination experiments as well as observations of pollen flow (J. Zimny et al., 2021), we conclude that for the variety 'Bogo,' an isolation distance of 11 m is sufficient to assure the 0.9% labeling threshold for food and feed required by Regulation (EC) 1829/2003 (unpublished results).

7. National Reference Laboratory for GMOs Supporting Official Control of GMOs in Poland and the EU

The experience gained in genetic modifications and molecular analyses of plants allowed us to establish the GMO Controlling Laboratory at the IHAR as an element of the biosafety system in Poland. In 2004, when Poland joined European Union, this laboratory became a member of the European Network of GMO Laboratories (ENGL). The latter is a consortium of official enforcement laboratories designated by the EU Member States. The primary objective of ENGL is to provide scientific and technical support to the European Union Reference Laboratory facing the challenges of proper detection, identification, and quantification of GMOs (Żmijewska et al., 2016; Żurawska-Zajfert et al., 2016). These tasks are required by Regulation (EC) 1829/2003. Plenary meetings are held annually to share the experiences gained by member countries. ENGL serves as a platform for technology transfer between

ENGL members and the Joint Research Center (JRC), promoting the exchange and training of scientists. ENGL experts have prepared guidance documents on topics of interest for executive laboratories (Corbisier et al., 2017; Trapmann et al., 2020). The GMO Controlling Laboratory at the IHAR was nominated as one of the National Reference Laboratories supporting the European Union Reference Laboratory for GM Food and Feed under Regulation (EC) 1829/2003 and the National Reference Laboratories under Regulation (EC) 2017/625. National Reference Laboratories collaborate with the JRC of the European Commission providing scientific and technical support in areas relevant to health and consumer protection (Żmijewska et al., 2017). The GMO Controlling Laboratory is accredited by the Polish Center for Accreditation according to the ISO 17025 standard, ensuring a high quality of analysis and flexible scope of accreditation.

8. New Genomic Techniques – Legal Problems and Challenges for Identification

We actively participate, through publications, lectures at scientific conferences, and as members of appropriate bodies, in the debate on the legal aspects of new genomic techniques in plant breeding, which takes place in the EU (Sowa et al., 2021; Twardowski et al., 2017; T. Zimny & Sowa, 2021; T. Zimny et al., 2019). Genome editing is becoming an efficient tool that is being increasingly used in plant breeding. However, in Europe, owing to legal restrictions, these new research methods are currently used exclusively for scientific purposes (Menz et al., 2020). After the judgment of the European Court of Justice (Eriksson & Zimny, 2020), which is widely interpreted as classifying products of most new genomic techniques as regulated GMOs, the European Commission decided that the current regulatory framework requires amendments to address problems that may be caused by the development and use of such products (European Commission, 2021).

Being aware of all these challenges, we continuously master genomic techniques through scientific research and must be ready to produce improved and safe breeding materials, now by optimizing the recent CRISPR/Cas9 method (Michalski et al., 2021).

9. Final Remarks

From the beginning, scientific work at the IHAR was carried out using the latest available methods and in cooperation with the best research centers in the country and abroad. Our colleagues continuously follow the ongoing events related to biotechnological innovations in the European market through active participation in both European (European Network for GMO Laboratories at the JRC, and EFSA Scientific Network for Risk Assessment of GMOs) and national bodies (Committee on Biotechnology of the Polish Academy of Sciences, Commission on GMM and GMO at the Ministry of Climate and the Environment, and Technical Committee of The Polish Committee for Standardization). Over the years, this has built up the authority of the institute and the department. We hope that this high-level of scientific research will be successfully continued.

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