Universität Rostock Traditio et Innovatio

Agrobiotechnology and Risk Assessment of Bio- and Gene Technology

Faculty of Agriculture and Environmental Science

Strategies to control mouse populations – production of species-specific contraceptive peptides in plants as oral vaccine

Cumulative Dissertation

Submitted for the Degree of

Doctor of Agricultural Sciences (doctor agriculturae, Dr. agr.)

Faculty of Agriculture and Environmental Science

University of Rostock

Submitted by

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Rostock, 2023

https://doi.org/10.18453/rosdok_id00004672

Universität Rostock Traditio et Innovatio

Professur für Agrobiotechnologie und Begleitforschung zur Bio-und Gentechnologie

Der Agrar- und Umweltwissenschaftlichen Fakultät

Strategien zur Kontrolle von Mäusepopulationen – Produktion artspezifischer kontrazeptiver Peptide in Pflanzen als oraler Impfstoff

Kumulative Dissertation

zur Erlangung des akademischen Grades

Doktor der Agrarwissenschaften (doctor agriculturae, Dr. agr.)

an der Agrar- und Umweltwissenschaftlichen Fakultät

der Universität Rostock

Vorgelegt von

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Rostock, 2023

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Jahr der Einreichung: 2023

Jahr der Verteidigung: 2023

WHEN YOU DO THINGS FROM YOUR SOUL,

YOU FEEL A RIVER MOVING IN YOU,

A JOY.

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Abbreviations

%	Percent
A.t.	*Agrobacterium tumefaciens*
AA	Amino Acid(s)
ANOVA	Analysis of variance
BSA	Bovine serum albumin
Con A	Concanavalin A lectin
C-terminus	Carboxy terminus
DNA	Deoxyribonucleic acid
dpi	Day post-infiltration
EB	Extraction buffer
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmatic reticulum
FITC	fluorescein isothiocyanate
GFP	Green fluorescent protein
GFP-mZP3-1	Fusion of GFP to mZP3-1 construct
HBcAg	Hepatitis B core antigen
HBcAg-mZP3	construct in which mZP3 peptide inserted in the e1 loop of HBcAg
His	Histidine
HPR	Horseradish peroxidase
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IMAC	Immobilized metal affinity chromatography
kDa	Kilodalton
KK	Dilysine linker
LB	Luria–Bertani medium
LDW	Leaf dry weight
MES	2-(N-morpholino) ethanesulfonic acid infiltration buffer
MIR	Major immunodominant region

mIzumo1	Mouse-specific peptide from sperm protein Izumo1
mIzumo1-3	Construct containing three copies of mIzumo1 peptide
mRNA	Messenger RNA
mZP2	Mouse-specific peptide from zona pellucida 2 protein
mZP2-3	Construct containing three copies of mZP2 peptide
mZP3	Mouse-specific peptide from zona pellucida 3 protein
mZP3-1	Construct containing a copy of mZP3 peptide
mZP3-3	Construct containing three copies of mZP3 peptide
N-terminus	Amino terminus
OD_{600}	Optical density measured at 600 nm wavelength
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
RT	Room temperature
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
TBM	Tetramethylbenzidine
TBS	Tris buffered saline
TBST	Tris buffered saline tween 20
T-DNA	Transfer DNA
TEM	Transmission electron microscopy
TMV	Tobacco mosaic virus
TSP	Total soluble protein
TT	T-cell epitope of tetanus toxoid
VLP	Virus like particle
ZP	Zona Pellucida

Summary

Rodents are a major problem in many parts of the world due to their high population densities. Traditional methods of rodent population control are non-specific and lethal methods that often pose a risk to humans, ecosystem, and non-target species. Therefore, it is imperative to develop a safe, more humane, and ethical approach to population reduction. Fertility control through immunocontraception can be a promising alternative. However, mice can only be vaccinated orally with immunocontraceptives. Oral vaccines must be species-specific to avoid effects on non-target organisms. Oral vaccines can be produced in plants inexpensively and on a large scale. Therefore, the aim of the present study was to develop strategies for the production of a mouse-specific and orally applicable contraceptive vaccine in plants.

The results demonstrated that mouse-specific epitopes of the contraceptive zona pellucida (ZP) proteins, ZP2 and ZP3, and the sperm protein Izumo were transiently produced as short peptides (mZP2, mZP3 and mIzumo1) in *Nicotiana benthamiana*. The stability and accumulation of peptides was significantly increased by fusion with stabilizing proteins or by trimerization. All three plant-produced peptides induced IgG antibodies in subcutaneously vaccinated mice. Moreover, oral delivery of the leaves expressing HBcAg-mZP3 fusion protein elicited systemic and mucosal immunity in mice without addition of an adjuvant. Mice immunized with a physical mixture of plant-produced mZP2-3 and mIzumo1-3 showed a 39 % reduction in the number of offspring. The litter size was negatively correlated with the level of antibodies against both mZP2 and mIzumo1. Induced anti- mZP3 and mZP2 antibodies recognized the native ZP and bound to the oocyte in wild mice ovaries *in vitro*. Notably, oral delivery of leaves expressing mZP3 peptide, in the form of HBcAg-mZP3, also elicited specific antibodies that cross-reacted with wild mice ZP. Observations suggest that antibodies may react with oocytes *in vivo* and reduce the fertility of wild-type mice.

In conclusion, we showed that small putative mouse-specific contraceptive peptides could be produced in sufficient quantities using a plant expression system. The generation of ZP-reactive antibodies by plant-produced peptides and the induction of antibody-correlated infertility in immunized mice warrant further study to investigate the contraceptive effect of plant-produced peptides in wild-type mice. The results of our experiments can be the basis for the development of mouse-specific contraceptive feeding baits for oral immunization of rodents and their population management.

Zusammenfassung

Nagetiere stellen auf Grund ihrer hohen Populationsdichte in vielen Teilen der Welt ein großes Problem dar. Herkömmliche Methoden zur Bekämpfung von Nagetierpopulationen sind unspezifisch und tödlich und bedeuten oft ein Risiko für Menschen, Ökosysteme und Nichtzielorganismen. Daher ist es unbedingt erforderlich, einen sicheren, humaneren und ethischeren Ansatz zur Reduktion der Populationen zu entwickeln. Die Fruchtbarkeitskontrolle durch Immunkontrazeption kann so eine Alternative sein. Ein Immunkontrazeptivum kann Mäusen aber nur oral verbreicht werden. Um einen Wirkung auf Nicht-Zielorganismen zu vermeiden müssen orale Impfstoffe artspezifisch wirken. Leider ist dies nur für kleinen Epitope der bisher zur Immunkontrazeption genutzten Proteine der Fall. Orale Impfstoffe lassen sich im großem Maßstab und kostengünstig in Pflanzen herstellen. Aufgabe der vorliegenden Arbeit war es daher Strategien zu entwickeln um in Pflanzen einen mausspezifischen, kontrazeptiven oral applizierbaren Impfstoff zu produzieren.

Mausspezifische Epitope der kontrazeptiven *Zona pellucida* (ZP) Proteine ZP2, ZP3 und des Spermienproteins Izumo wurden als kurze Peptide (mZP2, mZP3 und mIzumo1) in *Nicotiana benthamiana* transient produziert. Die Stabilität und Akkumulation der Peptide in Pflanzen wurde durch Fusion mit stabilisierenden Proteinen oder Trimerisierung deutlich erhöht. Alle drei in Pflanzen produzierte Peptide induzierten bei subkutan geimpften Mäusen die Bildung von IgG-Antikörper. Außerdem induzierte die orale Einnahme der Blätter, die das Fusionsprotein HBcAg-mZP3 exprimierten, ohne Adjuvans bei Mäusen eine systemische und mukosale Immunität. Mäuse, die mit einer Mischung aus in Pflanzen produziertem mZP2-3 und mIzumo1-3 immunisiert wurden, zeigten eine Reduzierung der Nachkommenzahl um 39 %. Die Wurfgröße korrelierte negativ mit der Menge von Antikörpern gegen mZP2 und mIzumo1. Induzierte Anti-mZP3- und mZP2-Antikörper erkannten die nativen ZP Proteine und banden *in vitro* an die Eizelle in den Eierstöcken von Wildmäusen. Bemerkenswert ist, dass auch die orale Verabreichung von Blättern, die das mZP3-Peptid in Form von HBcAg-mZP3 exprimierten, zur Bildung von Antiköropern führte die mit ZP von Wildmäusen kreuzreagierten. Unsere Beobachtungen legen nahe, dass Antikörper möglicherweise *in vivo* mit Eizellen reagieren und die Fruchtbarkeit bei Wildtyp-Mäusen verringern können.

Zusammenfassend haben wir gezeigt, dass kleine vermutliche mausspezifische kontrazeptive Peptide in ausreichenden Mengen in Pflanzen produziert werden können. Die Erzeugung von ZP-reaktiven Antikörpern durch pflanzenproduzierte Peptide und die Induktion von Antikörper-assoziierter Unfruchtbarkeit legt weitere Studien nahe, um den

Unfruchtbarkeitseffekt pflanzenproduzierter Peptide bei Wildtyp-Mäusen zu untersuchen. Die Ergebnisse der Experimente können die Grundlage für die Entwicklung mausspezifischer kontrazeptiver Futterköder zur oralen Immunisierung von Nagetiere und ihr Populationsmanagement sein.

1. Introduction

1.1 Rodent pest management

Human-wildlife conflicts are increasing worldwide due to overpopulation of wildlife, generally as a result of ecosystem disturbances (Polkinghorne et al., 2005). Invasive animal pest species have major negative effects such as environmental and ecological degradation. Rodents are the most important group of mammals in terms of the ecological damage and the major problems they cause. Worldwide, the rodents are the most agricultural, urban and social vertebrate pests (Barfield et al., 2006; Singleton et al., 2010). They reduce agricultural productivity and cause significant losses to agricultural products in the pre-harvest and post-harvest stages. Therefore, they can be one of the serious threats to food production in developed and developing countries (Singleton et al., 2007; Jacob, 2008). In Asia alone, rodents consume and waste crops that could feed about 200 million people every year (Stenseth et al., 2003). They disturb production and storage of crops by severe damage they directly and indirectly cause through consumption, spoilage, and contamination (Kaboodvandpour and Leung 2010; Wondifraw et al. 2021). They can also have major impacts in urban and rural habitats. Outbreaks of these pest species cause considerable losses in different parts of the world such as Australia (Brown and Henry, 2022), Africa (Swanepoel et al., 2017; Wondifraw et al., 2021), Europe (Singleton et al., 2007), and Asia (Singleton, 2010; Singleton et al., 2021). In addition, rodents can compromise the public's health by potentially carrying viruses, bacteria, pathogens and parasites; they play a significant role in transmission of a large number of diseases and pose a major risk to human health (Woolhouse et al., 2005; Firth et al., 2014). They can cause considerable damage and economic losses in livestock farms by transferring many different diseases, for example FMD (Food and Mouth Disease) or toxoplasmosis that threaten the whole livestock (Jacob, 2008).

The wide distribution and high reproductive capacity of rodents make control difficult. Current management methods are mostly based on lethal methods. Large-scale management using non-specific lethal methods such as poisoning, trapping, hunting, and explosives can cause environmental problems and poses direct and indirect risks non-target species (Elliott et al., 2016; Imakando et al., 2021). In addition, humans may be at risk when consuming contaminated food or handling rodenticides (Lefebvre et al., 2017). The current methods are not ethically accepted because they cause considerable suffering before the animal dies

(Jacob, 2008). Moreover, increases in reproductive rate may be observed in response to indiscriminate removal of animals (Nicholson and Bailey, 1935; Sinclair, 2003).

Concerns about human safety, animal welfare, and environmental impact necessitate more studies for the development of alternative control strategies (Gupta et al., 2011). To overcome the problem of overabundant wildlife populations, methods which are environmentally friendly and cost-effective need to be developed and promoted. Among the proposed environmentally friendly methods, fertility control is a promising non-lethal strategy to control invasive pests. Considering high reproductive rates and short life cycle of free-ranging rodent species such as mice, fertility inhibition could be a promising strategy to limit their populations (Tran and Hinds, 2013).

In this case, animals are not eliminated from the ecosystem and are not able to connect cause and effect to increase the fertility or avoid the baits/poisons (Barfield et al., 2006; Singleton et al., 2007). If fertility control can be shown to be effective, safe, and less harmful than its alternatives, it is the option of choice (Barfield et al., 2006). Immunocontraception which is a non-surgical method of fertility control is a feasible approach for small animals (Kirkpatrick et al., 2011; Massei and Cowan, 2014). The only practical way to reduce mouse populations through immunocontraception is oral vaccine bait (Yang et al., 2023). Notably, oral contraceptive baits have to be species-specific so as not to affect the fertility of non-target animals.

Immunocontraception is the method of inducing immune responses against proteins/hormones critical to the reproductive process (Chambers et al., 1997; Barber and Fayrer-Hosken, 2000). One of the main approaches that has been explored for development of contraceptive vaccines is inhibiting gamete function using vaccines based on oocyte- or spermatozoa-specific proteins (zona pellucida [ZP] proteins and sperm-based antigens) to prevent fertilization (Naz, 2005; Naz, 2006).

1.2 Gamete-specific proteins involved in reproductive process

Successful fertilization occurs through gamete recognition, specific sperm binding and penetration to the oocyte, and definitive polyspermy block (Hinsch et al., 1998). The zona pellucida (ZP) is an oocyte-specific protein with essential roles in the reproductive process (Barber and Fayrer-Hosken, 2000). The ZP is an extracellular glycoprotein matrix that surrounds the mammalian oocytes and ovulated eggs and is involved in different critical

stages of the fertilization (Wassarman and Litscher, 2008; Chiu et al., 2014). ZP is composed of three or four glycoproteins. In mice, ZP matrix consists of three glycoproteins known as ZP1, ZP2, and ZP3 (Bleil and Wassarman, 1980; Avella et al., 2013). The components have been named based on the molecular weight (Bleil and Wassarman, 1980; Spargo and Hope, 2003). For fertilization, sperm must recognize and bind tightly to ZP, penetrate and fuse with the egg plasma membrane following acrosome reaction (Bleil and Wassarman, 1980; Wassarman, 1988; Litscher and Wassarman, 2007). ZP2 and ZP3 glycoproteins represent the majority of ZP mass (Wassarman, 2008) and critical for formation and development of ZP, during the cascade of gamete recognition, and interactions (Modliński, 1970; Rankin et al., 2001; Wassarman et al., 2004; Avella et al., 2013).

ZP3 null mutant females mice have been shown to have oocytes and eggs lacking ZP and were infertile (Liu et al., 1996; Rankin et al., 2001). Variety of evidence has strongly suggested that ZP3 serves as the primary sperm receptor and the acrosome reaction inducer (Bleil and Wassarman, 1980; Kinloch et al., 1995; Wassarman et al., 2004). Evidence in mice suggest that ZP3 glycoprotein binds to the head of capacitated, acrosome-intact spermatozoa and subcutaneously induces the acrosome reactions in the sperm. Fertilization occurs only after the acrosome reaction, an exocytotic process that enables sperm to penetrate the ZP (Florman and Storey, 1982; Dean, 2004). ZP2 has been postulated to mediate secondary sperm binding to mammalian oocytes and to play an important role in sperm-ZP interaction and prevention of polyspermy. ZP2 functions as receptor for binding to the inner acrosomal membrane of acrosome-reacted sperm and thus penetration of sperm to the zona matrix (Bleil et al., 1988; Hinsch et al., 1998). Following fertilization, ZP glycoproteins are modified, probably by cortical granule enzymes, so ZP is no longer recognized by sperm, thus preventing sperm from binding to fertilized eggs or 2-cell mouse embryos (Bleil et al., 1981; Avella et al., 2014). Although the precise model of mouse sperm-egg binding interaction is controversial (Clark and Dell, 2006; Avella et al., 2013), there is extensive evidence demonstrating the critical roles and functions of ZP2 and ZP3 glycoproteins in fertilization. Figure 1.1A shows a schematic representation of mouse zona pellucida.

To achieve successful fertilization, sperm–egg fusion is the indispensable step, which is mediated by specific molecules from sperm side. The sperm-specific cell-surface protein Izumo1 has been shown to be the key molecule for the sperm–egg fusion in mice (Inoue et al., 2005). Acrosome is a subcellular organelle under the anterior plasma membrane of mouse sperm head (Avella and Dean, 2011). Izumo1 is detectable in the acrosomal membrane on the

mature sperm and at the time of acrosome reaction, translocated to the sperm surface (Figure 1.1B). Subsequently, the acrosome-reacted sperm penetrates the ZP and fertilizes the eggs (Inoue et al., 2005; Satouh et al., 2012; Bhakta et al., 2019). Izumo1-knockout male mice have been shown to be healthy but sterile. The sperm produced in the absence of Izumo1 (by Izumo1 $^{-/-}$ mice) bound to the zona pellucida but were incapable of fusing with the eggs. Furthermore, following the addition of antibodies against Izumo1, the sperms have not been able to fuse with the eggs (Inoue et al., 2005; Naz, 2006; Inoue et al., 2008). Therefore, the protein Izumo1 on spermatozoa is the factor that has been proven to be essential in sperm–egg fusion and fertilization.

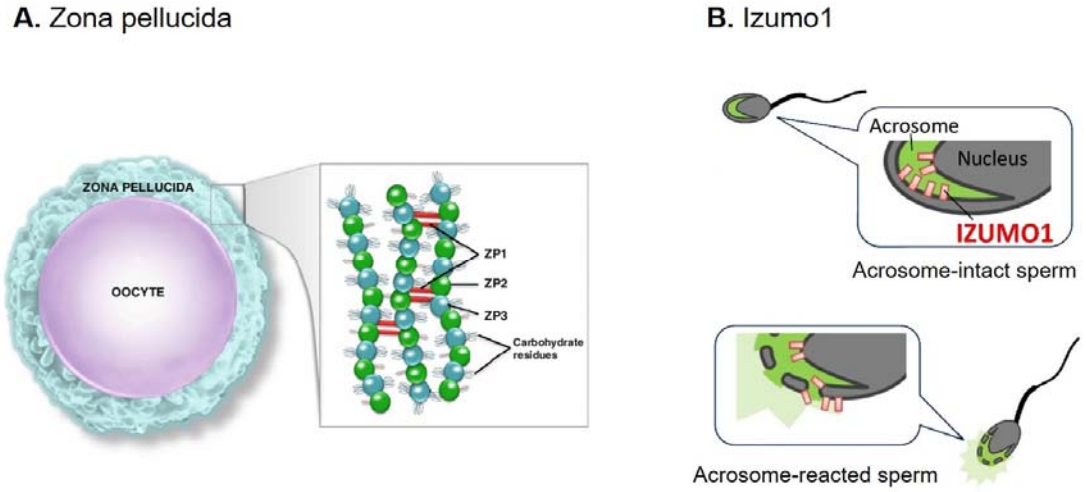

Figure 1.1: (A) The mouse zona pellucida (Dun et al., 2010). Fibrillary structure of zona pellucida, in which the major strands are composed of ZP2 and ZP3 glycoproteins. (B) Schematic representation of Izumo1 (Satouh and Ikawa, 2018), which is localized initially on the acrosomal membranes of acrosome-intact spermatozoa and translocated to the sperm surface after acrosome reaction.

1.3 Immunocontraception

Immunocontraception has been proposed as a valuable and acceptable method of population management. Immunocontraception is designed to interfere the biological activity of factors involved in different steps of reproductive process, resulting in inhibition of fertility. Several proteins involved in fertilization are being investigated for the development of contraceptive vaccines, including antigens associated with oocytes and sperm, as well as hormones and their receptor proteins [reviewed in (Naz and Saver, 2016; Gupta and Minhas, 2017)]. Contraceptive vaccines targeting gamete function include anti-oocyte and anti-sperm vaccines. Those gamete-specific antigens that have no unwanted interactions to other tissues,

are involved in fertility, and have the ability to induce antibodies preventing fertility can be employed for contraceptive vaccines (Naz, 2005; Naz and Saver, 2016).

Practical immunocontraceptive control of wildlife has been successful in some species at the field level. Since ZP glycoprotein plays crucial role in fertilization, it has been proposed as one of the most desirable targets for the development of contraceptive vaccines (Barber and Fayrer-Hosken, 2000; Naz, 2005). Due to their easy accessibility, porcine isolated ZP glycoproteins became the antigens of choice. Immunization with native porcine ZP proved to be non-species specific because it induces antibodies that prevents fertilization of numerous mammalian species. ZP proteins purified from porcine ovaries have been applied for regulation of white-tailed deer, grey seals, elks, bison, Feral horses, and elephants via injection [reviewed in (Naz and Saver, 2016; Gupta, 2022).

Due to the limitation in the amounts of native porcine ZP, high costs of preparation, and the risk of contamination by other ovarian-associated proteins, the development of recombinant ZP proteins has also been considered. Various research have investigated the production of recombinant ZP proteins and their effectiveness in preventing fertility. Recombinant ZP based contraceptive vaccines have been successful in inhibition of fertility in several animals such as bonnet monkeys, Koalas, baboons, Eastern Grey Kangaroos, and donkeys [reviewed in (Naz and Saver, 2016; Gupta and Minhas, 2017; Gupta, 2022).

Development of sperm-based vaccines have been also convincing. Sperm-specific protein Izumo1, which plays a vital role in sperm function, has been explored for immunocontraception. Antibodies generated against recombinant mouse Izumo1 almost completely prevented the fusion of mouse sperm and eggs *in vitro* (Wang et al., 2008). Immunization of mice with plasmid DNA containing the complete coding sequence of mouse Izumo1 resulted in significant reduction in fertilization rate and number of pups (An et al., 2009). In other research, a region of Izumo1 was identified that significantly inhibited the fusion of sperm with mouse egg and could induce a superior antifertility effect in vaccinated female mice (Wang et al., 2009; Xue et al., 2016). These results suggest that in addition to ZP proteins, Izumo1 can also be used as a suitable candidate for developing a contraceptive vaccine.

Most of the anti-fertility compounds used in wildlife management are injectable, requiring individual capture for treatment or remote delivery using darts (Chambers et al., 1997; Turner

et al., 2001; Jacoblinnert et al., 2021). The injectable contraceptive vaccines have been used for wild animals, including feral horses, elephants, elk, white-tailed deer, and bison [reviewed in (Barfield et al., 2006; Gupta, 2022). However, this approach is not practical for large-scale management of small free-ranging animals such as mice. For such animals, administration of vaccine through oral bait is the most suitable method of vaccine delivery. By incorporating the vaccine into an oral or mucosal formulation, administration of the vaccine to large populations of animals would be much more feasible and the cost will be considerably reduced (Naz and Saver, 2016; Faruck et al., 2021). However, since the complete ZP is not species specific, it can pose risks to non-target species exposed to the contraceptive baits. Because of evolutionary conserved sequences of the proteins, they have high grade of cross reactivity among mammals (Frank et al., 2005; Naz, 2005) and can cause infertility in other non-target species.

Instead of the whole protein, peptides corresponding to the epitopes that are less conserved between species and are crucial in fertility could be promising options. Targeting peptides from a regions of proteins that have undergone considerable sequence divergence during evolution is predicted to increase the target specificity of contraceptive vaccines as well as reduce the risk of side effects (Paterson et al., 2002; Hardy et al., 2004; Williams et al., 2006). A glycopeptide corresponding to 328–342 aa residues of mouse ZP3 has been suggested to be relevant for the biological activity of ZP3 with respect to sperm binding. Additionally, the peptide has not been detected immunologically in species such as guinea pig, hamster, cat, and dog (East et al., 1985; Millar et al., 1989). Antibodies against this peptide react with the epitopes on ZP3, lead to interference in biological activity resulting in inhibition of sperm-egg binding (Chen et al., 1998; Williams et al., 2006). Immunization of female mice with synthetic or recombinant forms of this peptide and other closely related peptides led to a reduction of fertility in some laboratory mouse strains and also wild mice (Millar et al., 1989; Lou et al., 1995; Hardy et al., 2002a). In other studies, a peptide of mouse ZP2 containing T- and B- cell epitopes, has been shown to have a role in sperm binding. Female mice immunized with ZP2 peptide have produced antibodies that reacted with mouse ovarian ZP and inhibited sperm-egg interaction (Sun, 1999; Hardy et al., 2008). Active immunization of different inbred mouse strains with ZP2 peptide elicited antibody with antifertility effect (Sun, 1999). An epitope peptide of mouse Izumo1, with low homology between various animal species, has been identified in the crucial Ig-domain of Izumo1, which is involved in binding

to the oocytes (Xue et al., 2016). Vaccine based upon the Izumo1 peptide caused a significant reduction in fertility of vaccinated mice (Naz, 2008).

Since these contraceptive peptide epitopes are poorly conserved between species, they are predicted to increase target specificity and reduce the possibility of unwanted side effects (Hinsch et al., 1999; Hardy et al., 2004; Hardy et al., 2008). The observations suggest that these putative mouse-specific peptides could be promising targets for the development of a contraceptive vaccine that would reduce the risk of cross-reaction with non-target species exposed to the vaccine baits in the field.

1.4 Production of contraceptive peptides

Principally, contraceptive peptides can be recombinantly produced by production systems ranging from prokaryotic systems such as *Escherichia coli* to eukaryotic systems such as yeast, insect and mammalian cells (Kusnadi et al., 1997; Ferrer-Miralles et al., 2009). Although bacterial expression systems are inexpensive and convenient, they are not able to produced posttranslationally modified proteins, moreover, the possess the risk of introducing toxins (Fischer and Emans, 2000; Burnett and Burnett, 2020b). Mammalian cells are capable of correct posttranslational modifications as an advantage over prokaryotic expression systems. However, they require complex and expensive culture, are difficult to scale-up, and have a high risk of pathogen contamination (Daniell et al., 2009; Schillberg and Finnern, 2021).

The other main disadvantage of these systems for oral vaccine production is that recombinant vaccines require processing and formulation into oral baits, which is difficult and expensive. However, plant production systems are potentially considerable cheap and safe platforms for the production of biopharmaceutical proteins and peptides, which can meet the requirements of oral vaccines (Giddings et al., 2000; Mason et al., 2002; Daniell et al., 2009). Advantages of plant production systems are mentioned in the following paragraphs.

1.5 Plant expression system

The field of plant molecular farming describes the production of pharmaceuticals and valuable products in plants (Moloney, 1995; Karg and Kallio, 2009). Human growth hormone expressed in transgenic tobacco in 1986 (Barta et al., 1986) was the first recombinant pharmaceutical protein made in plants (Ma et al., 2003). Since then, extending range of recombinant products including therapeutics, antibodies, vaccines, nutritional components,

and industrial enzymes have been produced in diverse range of crops (Schillberg and Finnern, 2021; Schillberg and Spiegel, 2022).

One of the major advantages of plant expression system is cost-effectiveness compared to traditional systems. Plants could provide attractive alternative system in terms of low production cost and low investment in infrastructure (Desai et al., 2010). Instead of expensive fermenters and bioreactors, plants can be grown in plant growth rooms, greenhouse, or in the field, which should reduce the upstream facility costs. It is estimated that cost of producing recombinant proteins in plants could be 2–10% and 0.1% of the cost of microbial fermentation and mammalian cell cultures, respectively, although this depends on the protein of interest, crop and product yield (Giddings et al., 2000; Twyman et al., 2003). Scalability is an important advantage for any expression systems, which is the limitation of fermentation and insect and mammalian cell expression systems. The plants offer cost-effective technologies that easily meet market demand compared to other systems (BOEHM, 2007; Davies, 2010). Production, harvesting and processing of materials on a large scale is possible using a plant expression system that has simple growth requirements and does not require large investments in hardware and culture media (Mason et al., 2002; Chan and Daniell, 2015). The speed of scale-up is also important. Unlike fermentation platforms, plants are effectively living bioreactors that can be scaled up simply by sowing more seeds. Moreover, one of the unique features of plant expression system is the diversity of species and plant systems used for production (Twyman et al., 2005; Schillberg and Finnern, 2021).

Proteins can be produced in plants using two systems: stable transformation and transient expression. Transient expression uses a recombinant plant virus that carries the gene of interest and causes the plant to express the gene. Advantage of these systems is the short development time and rapid onset of protein production, compared to time-consuming generation of stable transformation. The other advantages are ease of manipulation, low cost, high protein yield and scalability (Komarova et al., 2010). Transient expression is typically carried out in *N. benthamiana*, because of its transfection efficiency by *Agrobacterium* and plant viruses, as well as its growth pattern and high leaf biomass (Goodin et al., 2008; Holtz et al., 2015). However, it has also been reported for other *Nicotiana* species, potato, green pea, alfalfa, Arabidopsis and lettuce (Xu et al. 2012). The transient expression system based on a viral replicon mediated by *Agrobacterium tumefaciens* enables a rapid production of large amounts of recombinant protein in a short period of time (Komarova et al., 2010). Recombinant protein concentrations up to 80% TSP have already been achieved in *N.*

benthamiana using TMV-based MagnICON expression system (Marillonnet et al., 2004). This system combines the advantages of *Agrobacterium*-mediated DNA delivery and viral-vectors expression yield to provide rapid and high protein expression in a short period of time (Gleba et al., 2005; Komarova et al., 2010).

The cost of downstream processing such as extraction and purification plays a major role to determine the viability of a production system (Bosch and Schots, 2010; Xiao et al., 2016). Besides expensive production, the need for purification, cold storage/transportation, sterile delivery, and the short shelf life are the potential limitations of using recombinant proteins (Xiao et al., 2016). Since edible plants do not contain significant amounts of toxins or animal pathogens, plant tissues can be used in unprocessed or inexpensively processed forms for the development of oral vaccines (Twyman et al., 2003; Daniell et al., 2009). Thus, downstream processing costs would be significantly reduced when the plant expressing the recombinant protein are used directly as feed or food supplement (Kusnadi et al., 1997; Chan and Daniell, 2015). Furthermore, stable and relatively cheap storage of recombinant proteins in plant tissues, such as seeds, enables a cold-chain free and long-lasting storage. By eliminating the cost of expensive fermentation, purification, cold storage, and transportation, the recombinant proteins become affordable (Daniell et al., 2009). Plants are ideal for production of oral vaccines because the antigens can be protected by plant cell walls from acids and enzymes in the digestive system (Bio-encapsulation) (Kwon et al., 2013; Chan and Daniell, 2015; Xiao et al., 2016).

In addition, plant-derived products, whether purified or not, are less likely to be contaminated with human or animal pathogenic microorganisms. Hence they provide a safer alternative to other production systems for use as host systems for the production of desired proteins (Giddings et al., 2000; Kwon et al., 2013). In addition, they are free from toxins that could contaminate bacteria or mammalian cell culture preparations (Kwon et al., 2013). Therefore, producing therapeutic proteins in plants has also qualitative benefits by reducing the health risks caused by pathogen contamination.

Ability to perform posttranslational modifications such as protein glycosylation is another advantages of the plant production systems. This is a significant advantage over prokaryote expression systems when recombinant protein requires glycosylation to function properly. Glycosylation can affect properties of the recombinant protein such as protein folding, stability, solubility, and also immunogenicity (Kusnadi et al., 1997; Faye et al., 2005; Desai et

al., 2010). Despite some differences in post-translation processing between plant and mammals, they are few compared with other organisms (Ma et al., 2003; Bosch and Schots, 2010). Plant glycans may be beneficial in immunization by acting as adjuvants and enhancing the antigenicity and efficiency of plant-produced antigens (Bosch and Schots, 2010).

In some cases, plant systems may produce relatively low levels of recombinant proteins. However, the level of accumulation varies considerably between the recombinant products. There are examples of proteins with considerably high accumulation levels, which gives great promise for transgenic plants to be economically viable production systems (Kusnadi et al., 1997; Twyman et al., 2005; Buyel et al., 2017). Diverse approaches can be used to increase the yield of recombinant proteins produced by plants. Strategies such as optimizing the expression cassette or construct, stabilizing fusion partners, and targeting the recombinant protein to subcellular compartments have been shown to improve recombinant protein accumulation in plant (Benchabane et al., 2008; Karg and Kallio, 2009; Twyman et al., 2013).

1.6 Production of putative contraceptive peptides in plant

The knowledge about mouse-specific contraceptive peptides, mZP2, mZP3 and mIzumo1, combined with the expanded knowledge on plant expression system and plant-made vaccines, suggests that plant-based immunocontraceptive could become a viable strategy to control mouse population. Plants producing contraceptives can be administered orally and facilitate immunization of mouse populations (Naz and Saver, 2016). Reducing manufacturing costs is essential for the production of animal vaccines because the market will only tolerate labor-saving and cost-effective vaccines for animals (Floss et al., 2007; Schillberg and Finnern, 2021). Plant-based contraceptive vaccines can be economically produced and used directly as edible vaccines to avoid the cost of extraction and purification, and make such products much more economically competitive. Moreover, ability of plant glycosylation may be beneficial to produce the mZP3 peptide, since it is naturally glycosylated. The influence of glycosylation on the immunocontraceptive efficacy of ZP3-based antigens has already been reported. Glycosylation of ZP3 leads to a higher immunogenicity of mZP3 antigens compared to un-glycosylated or enzymatically/chemically de-glycosylated ones (Paterson et al., 1998; Hardy et al., 2003). Therefore, the plant production system can provide glycosylated mZP3 to be used for inducing immunological blocks to fertility.

However, production of small proteins is challenging and often ineffective (Molina et al., 2004). Small proteins and peptides are usually unstable since they are more susceptible to

proteases and can be rapidly degraded (Kusnadi et al., 1997; Demain and Vaishnav, 2009). Expression levels can be a major challenge specially for oral vaccines, since usually large amounts of the recombinant vaccines are required for oral delivery (Floss et al., 2007; Daniell et al., 2009). There are strategies to enhance expression level and stability of recombinant products, such as optimizing the codon usage for plant host (Streatfield, 2007; Lindgreen, 2012), targeting the expression of recombinant protein to specific subcellular such as endoplasmic reticulum (ER) (Gil et al., 2006; Aebi, 2013), and modifying the expression construct (Habibi et al., 2017). Moreover, expression of peptides as fusion proteins can significantly improve the accumulation levels of the target peptides (Molina et al., 2004; Floss et al., 2007). The fusion strategy using stabilizing partners, such as GFP, is one of the proven methods to improve the synthesis, stability, and accumulation of recombinant proteins in plants (Streatfield, 2007; Benchabane et al., 2008; Hsieh et al., 2010).

Although the development of peptide-based contraceptives can improve specificity and safety of vaccines, they are generally less effective than whole protein vaccines (Ferro and Mordini, 2004; Hardy et al., 2008). However, combining repeated contraceptive peptides in an antigen can improve the immunogenicity and effectiveness of the antigen in reducing fertility compared to a single- peptide antigen (Hardy et al., 2004; Redwood et al., 2007; Mortazavi et al., 2021). In addition, an immunogen containing a mixture of contraceptive epitopes of ZP and Izumo proteins can also enhance vaccine efficacy by inducing antibodies against multiple targets (Choudhury et al., 2009; Shrestha et al., 2014; Gupta et al., 2022). Fusion of epitopes to immune-enhancing carriers, such as virus like particles (VLPs), is also a promising approach to improve the immunogenicity of weak antigens (Blokhina et al., 2013; Chen and Lai, 2013).

To investigate the ability of plant to produce small contraceptive peptide, the MagnICON-based transient expression system is suitable as alternative to the time consuming transgenic approach. The expression of small mouse-specific peptides needs to be evaluated and optimized. Therefore, transient expression of peptides in *N. benthamiana* would be useful to achieve the objectives in shorter time.

1.7 Research objectives

Since the development of an oral mouse-specific contraceptive vaccine is necessary to control the mouse population, the present dissertation project was carried out with the aim of discovering the capability of plant to produce small mouse-specific contraceptive peptides. In

addition, the plant-produced peptides will be used in immunization of female BALB/c mice to determine their effectiveness in inducing antibody responses and infertility.

More specific objectives were:

- To evaluate the capability of plants for production of small contraceptive peptides using *Agrobacterium*-mediated MagnICON-based transient expression in *N. benthamiana*.
- To optimize the expression of peptides in plants using fusion partners and construct modifications.
- To evaluate the capability of plant-produced small peptides to induce antibody responses, through subcutaneous injection, and to determine the most immunogenic antigen.
- To examine mucosal and systemic immune responses after oral administration of selected antigen.
- To evaluate the effect of plant-produced peptides on the fertility of immunized mice.
- To assess the *in vitro* interaction of induced antibodies to wild mice oocyte using immunohistochemistry.

2. Results

The results of this work are presented in three articles published in peer-reviewed scientific journals.

2.1. Plant-Produced Mouse-Specific Zona Pellucida 3 Peptide Induces Immune Responses in Mice.

2.2. Oral and Subcutaneous Immunization with a Plant-Produced Mouse-Specific Zona Pellucida 3 Peptide Presented on Hepatitis B Core Antigen Virus-like Particles.

2.3. Immunogenicity and contraceptive efficacy of plant-produced putative mouse-specific contraceptive peptides.

2.1 Plant-Produced Mouse-Specific Zona Pellucida 3 Peptide Induces Immune Responses in Mice

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Published in

Vaccines (2023), 11(1), 153.

DOI: https://doi.org/10.3390/vaccines11010153

vaccines

MDPI

Article

Plant-Produced Mouse-Specific Zona Pellucida 3 Peptide Induces Immune Responses in Mice

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Abstract: Contraceptive vaccines are designed to stimulate autoimmune responses to molecules involved in the reproductive process. A mouse-specific peptide from zona pellucida 3 (mZP3) has been proposed as a target epitope. Here, we employed a plant expression system for the production of glycosylated mZP3 and evaluated the immunogenicity of plant-produced mZP3-based antigens in a female BALB/c mouse model. In the mZP3-1 antigen, mZP3 fused with a T-cell epitope of tetanus toxoid, a histidine tag, and a SEKDEL sequence. A fusion antigen (GFP-mZP3-1) and a polypeptide antigen containing three repeats of mZP3 (mZP3-3) were also examined. Glycosylation of mZP3 should be achieved by targeting proteins to the endoplasmic reticulum. *Agrobacterium*-mediated transient expression of antigens resulted in successful production of mZP3 in *Nicotiana benthamiana*. Compared with mZP3-1, GFP-mZP3-1 and mZP3-3 increased the production of the mZP3 peptide by more than 20 and 25 times, respectively. The glycosylation of the proteins was indicated by their size and their binding to a carbohydrate-binding protein. Both plant-produced GFP-mZP3-1 and mZP3-3 antigens were immunogenic in mice; however, mZP3-3 generated significantly higher levels of serum antibodies against mZP3. Induced antibodies recognized native zona pellucida of wild mouse, and specific binding of antibodies to the oocytes was observed in immunohistochemical studies. Therefore, these preliminary results indicated that the plants can be an efficient system for the production of immunogenic mZP3 peptide, which may affect the fertility of wild mice.

Keywords: wildlife population control; immunocontraception; zona pellucida 3; recombinant protein; plant-produced vaccine; *Nicotiana benthamiana*

Citation: Ghasemian, K.; Broer, I.; Schön, J.; Kolp, N.; Killisch, R.; Huckauf, J. Plant-Produced Mouse-Specific Zona Pellucida 3 Peptide Induces Immune Responses in Mice. *Vaccines* **2023**, *11*, 153. https://doi.org/10.3390/vaccines11010153

Academic Editors: François Meurens, Fanny Renois and Jianzhong Zhu

Received: 8 December 2022
Revised: 4 January 2023
Accepted: 6 January 2023
Published: 10 January 2023

1. Introduction

Rodents are among the most important vertebrate pests in many parts of the world, causing economic, social, and environmental costs and damage [1,2]. Mice, in particular, are considered serious agronomic pests both in the preharvest and postharvest stages [3,4]. Moreover, they can endanger public health by spreading diseases to humans [1,5,6]. Therefore, controlling rodent pest species is an important issue. The most common control techniques rely on lethal methods such as poison baiting and trapping [7]. Conventional control methods are associated with concerns about safety, specificity, animal welfare, and environmental impact. Consequently, there has been growing interest in developing more humane techniques that are cost-effective, species-specific, and environmentally friendly to control mouse populations [8].

In this regard, fertility control is an interesting alternative for controlling vertebrate pests, including mice [8–11]. Contraception by induced immunity (immunocontraception) is a fertility regulation method using the immune system to disrupt reproductive function and prevent fertilization [9,12,13]. Contraceptive vaccines evoke an immune response to molecules that have indispensable roles in the reproduction process. Several proteins have

been used successfully to reduce the fertility of various animal species, including antigens associated with sperm [14–16] and oocytes [17–19], as well as hormones and their receptor proteins [20,21]. The unique specificity of sperm and oocyte proteins makes them ideal candidates for immunocontraceptive vaccines. The oocyte-specific protein zona pellucida (ZP), with a pivotal role in the process of fertilization, has been proposed as one of the most desirable targets for the development of contraceptive vaccines [13,22]. The ZP is a glycoprotein matrix that surrounds the plasma membrane of all mammalian oocytes. In mammals, the ZP is composed of either three or four glycoproteins. Antigens derived from the ZP protein subunits have successfully induced an immunocontraceptive effect in various species of mammals [12,23]. In mice, the ZP matrix consists of three glycoproteins conventionally known as ZP1, ZP2, and ZP3 [24–26]. ZP3 protein-based antigens have been reported to have a strong contraceptive effect and lead to reduced fertility in many species [27–29]. However, the lack of species specificity of protein-based vaccines limits their widespread use for wildlife population control [13,30]. Moreover, injectable immunocontraceptives are clearly not suitable for field applications for controlling the population of small- and large-scale dispersed animals such as wild mice. The availability of oral immunocontraceptive vaccines could increase the scope of contraceptive applications for wildlife population control and facilitate mouse population management.

Oral application of vaccine necessitates species specificity of contraceptive antigens to avoid affecting the fertility of non-target animals. The development of peptide-based contraceptive vaccines is an attractive approach for producing species-specific antigens [31–33]. Targeting rodent-specific peptides from areas of ZP proteins that are less conserved between species has been predicted to increase the target specificity of contraceptive vaccines [34–36]. In mice, the ZP3 protein plays a key role during fertilization as the primary receptor for sperm and the inducer of the sperm acrosome reaction [37–39]. A short glycopeptide of ZP3 corresponding to amino acid residues 328–342 (mZP3) has been suggested to be directly involved in these activities [38,40]. Vaccines based on the mZP3 contraceptive peptide appear to have the desired properties. First, immunization of mice with synthetic or recombinant forms of this peptide and other closely related peptides has reduced fertility in some mouse strains, including wild mice [15,41–43]. Second, it has not been detected immunologically in other species such as hamster, guinea pig, cat, or dog [41,44]. Therefore, the mZP3 peptide is considered a promising target for developing a mouse-specific contraceptive vaccine [32,43]. However, in many cases, peptide-based vaccines have lower immunogenicity than protein antigens [45]. Integrating adjuvants—such as a 'promiscuous' T-cell epitope from tetanus toxoid, which is reported to be a universal immunogenic epitope—is an approach towards improving the efficacy of peptide-based vaccines [46–49]. Furthermore, the use of repeated peptide epitopes has been shown to enhance the immunogenicity of contraceptive peptide-based vaccines [32,35,50].

Transgenic plants are a promising platform for producing oral vaccines. Plants offer several advantages compared to other available conventional systems, such as bacterial and mammalian cell-based production systems. Plant expression systems are considered rapid, cost-effective, and safe platforms for the large-scale production and delivery of vaccines [51,52]. In addition, plants have the ability to produce functional proteins with eukaryotic post-translational modifications including glycosylation, which are often required for the biological activity and function of many mammalian proteins [53,54]. Furthermore, plant expression systems provide safety benefits, as they greatly reduce the risk of contamination and transmission of mammalian pathogens from the host [55,56]. Nevertheless, the expression of small proteins can be a challenge to eukaryotic expression systems [57]. Several studies have illustrated the positive effect of protein fusion as a strategy to improve the production of recombinant proteins in plant production systems [58].

Plant-derived recombinant proteins can be expressed either by stable transformation or through transient expression. The latter approach provides for rapid production of high yields of recombinant proteins compared to time-consuming stable transformation [59]. MagnICON technology is a transient expression system based on a plant viral vector, with

the potential of producing recombinant protein up to 80% of total soluble protein [60]. This system combines the advantages of different biological systems, including the efficient DNA delivery of *Agrobacterium tumefaciens*, the speed and high expression levels of a plant RNA virus, as well as posttranslational modifications and low cost of plant expression systems [61,62].

In this study, we explored the potential of plants for producing glycosylated putative contraceptive antigens using the MagnICON expression system in *N. benthamiana*. We chose a mouse-specific glycopeptide from ZP3 (mZP3, amino acids 328–342) as the contraceptive epitope for constructing the antigens. The T-cell help for eliciting an immune response was provided by inclusion of T-cell epitope from tetanus toxoid (TT). The objectives of this study were to evaluate the production of glycosylated mZP3-based antigens in *N. benthamiana* and to evaluate the capability of this small peptide to induce antibody responses by immunization with plant-produced antigens. Immunohistochemistry was performed using ovarian sections of wild mouse to assay antibody–oocyte interaction.

2. Materials and Methods

2.1. Construction of Plant Expression Vectors

The coding sequences of mZP3-1 (containing a copy of mZP3 peptide) and mZP3-3 (containing three copies of mZP3 peptide) constructs were designed and codon optimized for expression in *N. benthamiana* (Figure S1). The gene constructs were synthesized by Eurofins Genomics GmbH (Ebersberg, Germany) and cloned into the pEX-A vector. To construct GFP-mZP3-1, the green fluorescent protein (GFP) coding region was amplified from the vector pICH18711 [60] and fused to the 3′ end of the mZP3-1 by overlapping PCR. All coding regions were flanked by BsaI restriction sites using PCR amplification. The PCR products were cloned separately into the pJET1.2 cloning vector (CloneJET PCR Cloning Kit, Thermo Fisher Scientific, Waltham, MA, USA). Subsequently, the gene sequences were inserted into the TMV-based expression vector pICH31120 of the MagnICON system with BsaI restriction/ligation [63]. The MagnICON™ vectors were kindly provided by Nomad Bioscience (Halle/Saale, Germany). The binary MagnICON vector pICH31120 and the expression constructs are shown in Figure 1.

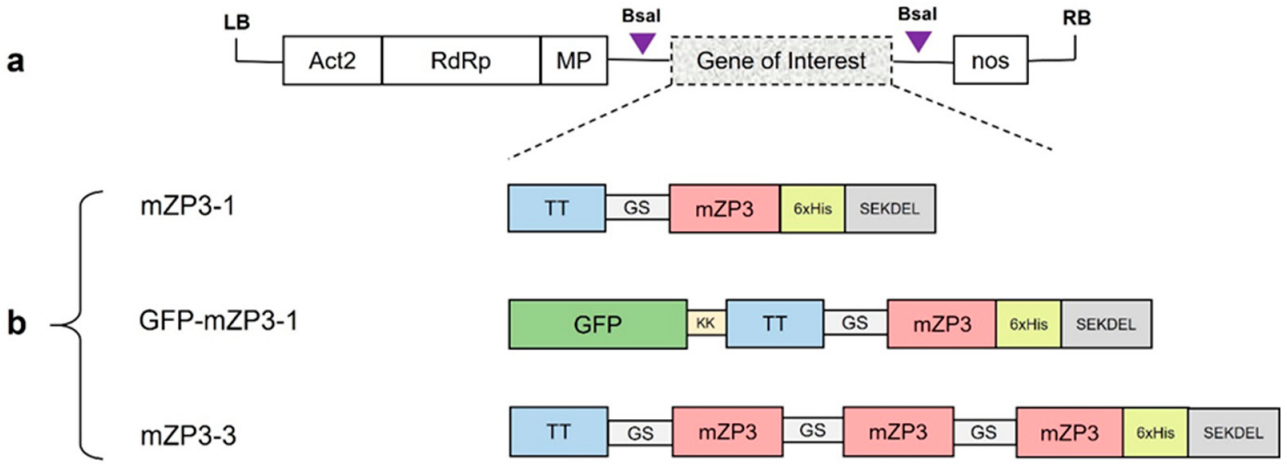

Figure 1. Schematic representations of the T-DNA of MagnICON and the expression constructs used for transient expression of the contraceptive mouse ZP3 peptide in the plant. (**a**) Binary 'MagnICON' vector used for transient expression. Act2, Arabidopsis actin 2 promoter; RdRp, RNA-dependent RNA polymerase; MP, TMV movement protein; nos, *A. tumefaciens* nopaline synthetase gene terminator. Triangles represent BsaI cleavage sites. (**b**) mZP3-1, GFP-mZP3-1, and mZP3-3 constructs used for transient expression. mZP3, mouse ZP3 peptide (aa residues 328–342) contains T-cell and B-cell epitopes; TT, T-cell epitope of tetanus toxoid (aa residues 830–844); GS, (GSSSS)3 flexible linker; 6xHis, 6 histidine residues; SEKDEL, ER retrieval signal; KK, dilysine linker.

2.2. Agroinfiltration Procedure

Expression vectors with their cassettes were separately electroporated into *Agrobacterium tumefaciens* strain ICF320. Transformed colonies were inoculated into 5 mL Luria–Bertani (LB) media supplemented with 50 µg/mL kanamycin and 50 µg/mL rifampicin and incubated overnight at 28 °C in a shaking incubator (220 rpm). Two milliliters of the *Agrobacterium* suspensions were diluted into 200 mL cultures containing the same antibiotics and grown at 28 °C/220 rpm overnight. The *Agrobacterium* cells were harvested by centrifugation at 4560× *g* for 30 min at room temperature (RT) and resuspended in infiltration buffer (10 mM 2-(N-morpholino) ethanesulfonic acid (MES), pH 5.8, 10 mM $MgSO_4$, 0.02% Silwet Gold) to a final OD_{600} of 0.15–0.2. Transient expression was carried out by vacuum-agroinfiltration of 6- to 8-week-old *N. benthamiana* grown in the greenhouse as described previously [64].

2.3. Total Soluble Protein Extraction from N. benthamiana Leaves

Agroinfiltrated *N. benthamiana* leaves were harvested at various days post-infiltration (dpi), lyophilized using a freeze dryer system and ground. Total soluble protein (TSP) was extracted from freeze-dried leaf material (25 mg) homogenized in 500 µL ice-cold extraction buffer using a Precellys 24 homogenizer (Bertin Instruments, Montigny-le-Bretonneux, France). The extract was clarified by repeated centrifugation at 15,000× *g* for 15 min at 4 °C. The supernatant was collected as the total soluble protein (TSP) fraction, and the protein concentration was measured using the Pierce™ Coomassie Plus (Bradford) assay kit (Thermo Fisher Scientific), with bovine serum albumin (BSA) as the standard.

2.4. Ni-NTA Purification

His-tagged recombinant mZP3-1, GFP-mZP3-1, and mZP3-3 were purified using immobilized metal affinity chromatography (IMAC) based on the affinity for the 6His purification tag. For large-scale protein extraction, 30 g of ground leaf material was homogenized in ice-cooled protein extraction buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM sucrose, 5 mM imidazole) using a Homogenizer (Polytron® PT 2100, Kinematica AG, Malters, Switzerland) for 30 s at 30,000 rpm. Lysates were centrifuged at 4600× *g* for 15 min and then clarified by repeated ultracentrifugation at 16,000× *g* for 30 min at 4 °C. The supernatants were collected and loaded onto a column (Bio-Rad Laboratories, Hercules, CA, USA) containing pre-equilibrated Nuvia™ IMAC Ni-charged resin (Bio-Rad Laboratories, Hercules, CA, USA). The column was washed with washing buffer (50 mM NaH_2PO_4, 20 mM imidazole, 20 mM NaCl, pH 8). The target recombinant protein was further eluted with elution buffer (50 mM NaH_2PO_4, 300 mM imidazole, 300 mM NaCl, pH 8). The elution fraction was concentrated and desalted using Vivaspin 20 centrifugal concentrators with a 10 kDa cut-off membrane (Sartorius AG, Göttingen, Germany).

2.5. SDS–PAGE and Western Blot Analysis

Protein extracts were mixed with loading buffer (150 mM Tris, pH 6.8, 10% glycerin, 3% SDS, 1% β-mercaptoethanol, and 2.5% bromophenol blue) and denatured at 95 °C for 5 min. The proteins were separated under denaturing conditions using 12% SDS–PAGE and then electroblotted onto a nitrocellulose membrane (GE Healthcare Europe GmbH, Solingen, Germany) via wet transfer at 100 V for 30 min. Membranes were blocked for 2 h with 5% (*w/v*) nonfat milk powder in TBST, pH 7.6, containing 20 mM Tris, 150 mM NaCl, and 0.05% (*v/v*) Tween 20. After three washes with TBST, membranes were probed with mouse monoclonal anti-His antibodies (Dianova, Hamburg, Germany) (1:1000 dilution) for 2 h at RT. Following another washing step, the membranes were probed with horseradish peroxidase (HRP)-conjugated donkey anti-mouse antibodies (Dianova) (1:10,000 dilution) for 1 h at RT. After a final wash with TBS, proteins were detected with the enhanced chemiluminescence technique using a Kodak Biomax light X-ray film (VWR; Darmstadt, Germany). Concanavalin A (Con A) lectin blot analysis was conducted as previously described [64] to visualize the glycosylation of recombinant proteins.

2.6. Enzyme-Linked Immunosorbent Assay (ELISA)

The content of mZP3 peptide was determined using ELISA in 96-well microtiter plates. Briefly, the plate was coated with diluted leaf extract in carbonate buffer (pH 9.6) for 2 h at RT and washed three times with PBS containing 0.05% Tween 20 (PBST). The plate was blocked with 1X RotiBlock blocking solution (Carl Roth GmbH + Co. KG, Karlsruhe, Germany) for 1 h at RT and washed as previously described. Rabbit anti-mZP3 primary antibody (Bioserv, Rostock, Germany) was added at a 1:500 dilution in PBS, incubated at RT for 2 h, and then washed three times. The plate was then incubated for 1 h at RT with HRP-conjugated goat anti-rabbit secondary antibodies (Dianova) at a 1:2000 dilution in PBS. Tetramethylbenzidine (TMB) substrate was added to each well to develop the color in the dark for 10 min, and the reaction was stopped with 250 mM H_2SO_4. Absorbance was read at 450 nm.

2.7. Subcutaneous Immunization of Mice

The animal experiments were performed in accordance with the German animal protection regulations and were approved by the relevant authorities (Landesamt fur Landwirtschaft, Lebensmittelsicherheit und Fischerei, Mecklenburg-Vorpommern, Germany; permission No. 7221.3-1-073/17 and 7221.3-3.2-002/22).

Female BALB/c mice (6–8 weeks old) were divided into three groups with five mice in each group. Primary immunization was carried out on day 1 and two booster immunizations at three-week intervals. Injections were administered over the mouse neck. The first group received GFP-mZP3-1 (containing 50 μg mZP3), the second group received mZP3-3 (containing 50 μg mZP3), and the control group received PBS. All doses contained 10% Polygen (MVP Lab) as an adjuvant. Serum samples were collected before primary immunization and two to three weeks after each immunization. Female BALB/c mice were mated with proven fertile BALB/c males of similar age three weeks after the final immunization. Males were removed after 2 weeks, and the females were allowed to litter.

2.8. Detection of the Antibody Titer Using ELISA

The specific IgG responses in serum were determined using endpoint titer ELISA. Briefly, a 96-well plate was coated with 50 μL of plant-produced mZP3 (0.5 μg per well) and incubated at RT for 2 h. Nonspecific binding was prevented by incubation in PBS buffer containing 1% (*w/v*) BSA for 1 h at RT. The plates were washed three times with PBST and incubated with serum samples (50 μL per well) serially diluted in PBS at RT for 2 h. Then, the plate was incubated with HRP-conjugated donkey anti-mouse secondary antibodies (Dianova, Hamburg, Germany) at a dilution of 1:2000 in PBS at 37 °C for 1 h. The plate was washed, and the color was developed with TMB substrate solution (100 μL per well). After 12 min, the reaction was terminated by adding 100 μL of 250 mM H_2SO_4. Absorbance values were determined at 450 nm.

2.9. Indirect Immunofluorescence

Mouse ovarian sections were provided by BIOSERV Analytik (Rostock, Germany). Sections were deparaffinized and subjected to antigen retrieval using sodium citrate buffer (10 mM sodium citrate, 0.05% Tween 20, pH 6.0). Sections were blocked with 10% goat serum blocking solution (Life Technologies, Frederick, MD, USA) at RT for 1 h, rinsed with PBS, and incubated in 1× mouse-on-mouse IgG blocking solution (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) at RT for 1 h. Sections were incubated with a 1:30 dilution of serum samples and kept at 4 °C overnight. The sections were washed and incubated with 10 μg/mL fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Invitrogen) as a secondary antibody for 1.5 h at 37 °C. After washing, the slides were mounted with DAPCO mounting medium (25 mg/mL DABCO, 90% glycerol, 10% PBS, pH 8.5) and observed under a fluorescence microscope. The sections were stained with methylene blue for bright-field microscopy.

2.10. Histological Analysis

On day 99 of the experiment, one mouse from each group was sacrificed. The ovaries were collected, fixed in 4% paraformaldehyde and then paraffin embedded. The ovaries were sectioned at 5 µm, stained with hematoxylin and eosin, and observed under a microscope.

2.11. Statistical Analysis

The experimental data were analyzed using IBM SPSS statistical software version 27. The comparison of means was performed using one-way analysis of variance (ANOVA). Duncan's test was used as post-hoc test to measure specific differences between the means. The statistically significant differences are shown with one asterisk (*), indicating p values < 0.05, and two asterisks (**), indicating p values < 0.001.

3. Results

3.1. Construction of Expression Vectors for Transient Expression

The mouse zona pellucida 3 peptide CSNSSSSNSSSSQFQ (mZP3), corresponding to amino acids 328–342, contains a T-cell and a B-cell epitope [41–43]. To assess the expression of mZP3 in plants and immunogenicity of plant-made recombinant mZP3 in mice, three gene constructs (mZP3-1, GFP-mZP3-1, and mZP3-3) were developed (Figure 1b). In the mZP3-1 construct, a promiscuous T-cell epitope (aa residues 830–844) of TT was fused at the N-terminus of the mZP3 sequence using a flexible GS linker to enhance the immunogenicity of the antigen [48]. To facilitate the purification of recombinant protein, a 6×His tag was fused at the C-terminal end of mZP3. The retention in the endoplasmic reticulum (ER) was achieved through the addition of a SEKDEL retention signal at the C-terminus of the construct. The GFP-mZP3-1 construct was developed by fusing the mZP3-1 construct to GFP via a dilysine linker (KK). In the mZP3-3 construct, the antigenic peptide was tripled by linking three repeated copies of mZP3 via GS linkers. All three resulting constructs were codon optimized for *N. benthamiana* to increase protein expression and cloned into the MagnICON-based plant expression vector pICH31120 to target the expression of proteins into the ER.

3.2. Optimization of mZP3 Expression in N. benthamiana

N. benthamiana plants were infiltrated with the resulting vectors in three independent repetitions. Protein expression was examined following transient expression in *Agrobacterium*-infiltrated leaves. The leaves were harvested at different days post-infiltration (dpi), and total soluble proteins were extracted and determined with a Bradford assay. Time-course evaluation of protein expression levels of each construct was performed using western blot probed with anti-His monoclonal antibodies. The optimal harvest time of recombinant proteins was determined based on the accumulation of target proteins and morphology of the infiltrated leaves. Western blot analysis of TSP from leaves infiltrated with mZP3-1 showed no protein band corresponding to the expected size of ~8 kDa for the mZP3-1 protein (Figure S2). Accordingly, no band at the expected size for the non-glycosylated forms of GFP-mZP3-1 and mZP3-3 (34.7 and 11 kDa, respectively) could be detected (Figure 2). However, a strong band representing a protein with a molecular mass of ~37 kDa was detected in the total soluble protein extracts from leaves infiltrated with GFP-mZP3-1. Protein accumulation was observed in all selected leaves, reached the highest level at 8 dpi, and gradually declined thereafter (Figure 2, left). In western blot analysis of crude extract from mZP3-3-expressing leaves, a ~18 kDa protein band was detected. Accumulation of the protein was observed from 5 dpi to 7 dpi, while the protein was barely detectable before and after this time (Figure 2, right). A higher molecular mass band (~36 kDa) was also presented, which appeared to be the dimer form of the protein.

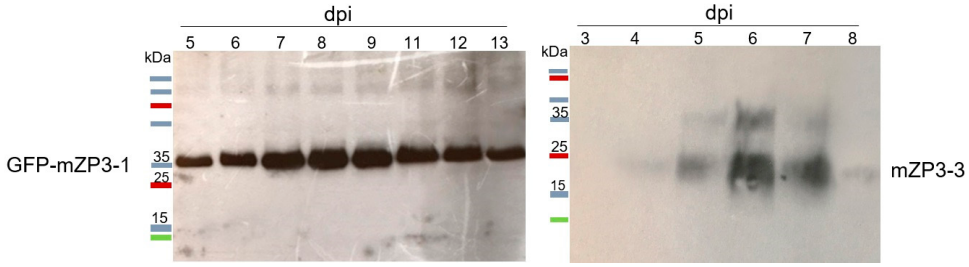

Figure 2. Western blot of *N. benthamiana* crude leaf extracts to detect protein expression and to determine the optimal harvest time. Expression of GFP-mZP3-1 (**left**) and mZP3-3 (**right**) in the infiltrated leaves, detected by western blot of 30 µg TSP, probed with anti-His monoclonal antibodies. dpi, days post-infiltration. Blots cropped for clarity, for uncropped blots see Figure S3.

The plant-produced proteins showed higher molecular masses than those expected for unglycosylated proteins. This finding pointed to the possible addition of glycan chains during expression in plants and retention in the ER, since the mZP3 peptide carries two potential glycosylation sites. The presence of glycans on the plant-produced GFP-mZP3-1 and mZP3-3 proteins was assessed with lectin blotting using peroxidase-conjugated concanavalin A (Con A), which has a high affinity for binding to sugar residues. Lectin blots of recombinant GFP-mZP3-1 and mZP3-3 probed with Con A identified carbohydrates and revealed dominant bands of ~37 kDa and ~18 kDa, respectively (Figure 3). This result indicated that the observed protein bands corresponded to glycosylated forms of the GFP-mZP3-1 and mZP3-3 recombinant proteins.

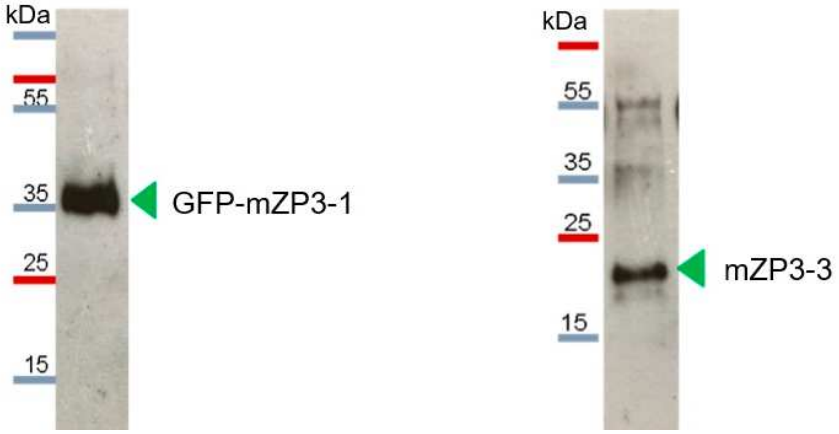

Figure 3. Profile of lectin binding to the *N. benthamiana*-expressed proteins to detect the presence of sugar residues on proteins. The profile represents the binding of Con A to the recombinant GFP-mZP3-1 (**left**) and mZP3-3 (**right**). Lectin blot labeled with peroxidase-conjugated concanavalin A. Blots cropped for clarity, for uncropped blots see Figure S4.

Protein analysis showed that GFP-mZP3-1 and mZP3-3 targeted to the ER were successfully produced in *N. benthamiana* plants. Based on the plant morphology and highest accumulation of proteins in plants, the optimal harvest time for GFP-mZP3-1- and mZP3-3-infiltrated leaves was determined to be approximately 8 dpi and 6 dpi, respectively.

All infiltrated plants displayed morphological disorders from chlorosis to necrosis and finally leaf death, although at different dpi. In the case of mZP3-1, necrosis started on day 3 and increased rapidly, resulting in plant death by day 7 (Figure 4a). However, when the plants were infiltrated with the GFP-mZP3-1 fusion construct, the phenotypic effects were less pronounced, and symptoms of chlorosis began on day 7 (Figure 4b). In plants infiltrated with mZP3-3, leaves started to show necrosis at 4 dpi. Wilting was observed on day 7 and resulted in plant death by day 10 (Figure 4c). Expression of mZP3-3 caused

moderate leaf necrosis compared to mZP3-1, although it was more severe than that of GFP-mZP3-1 expression.

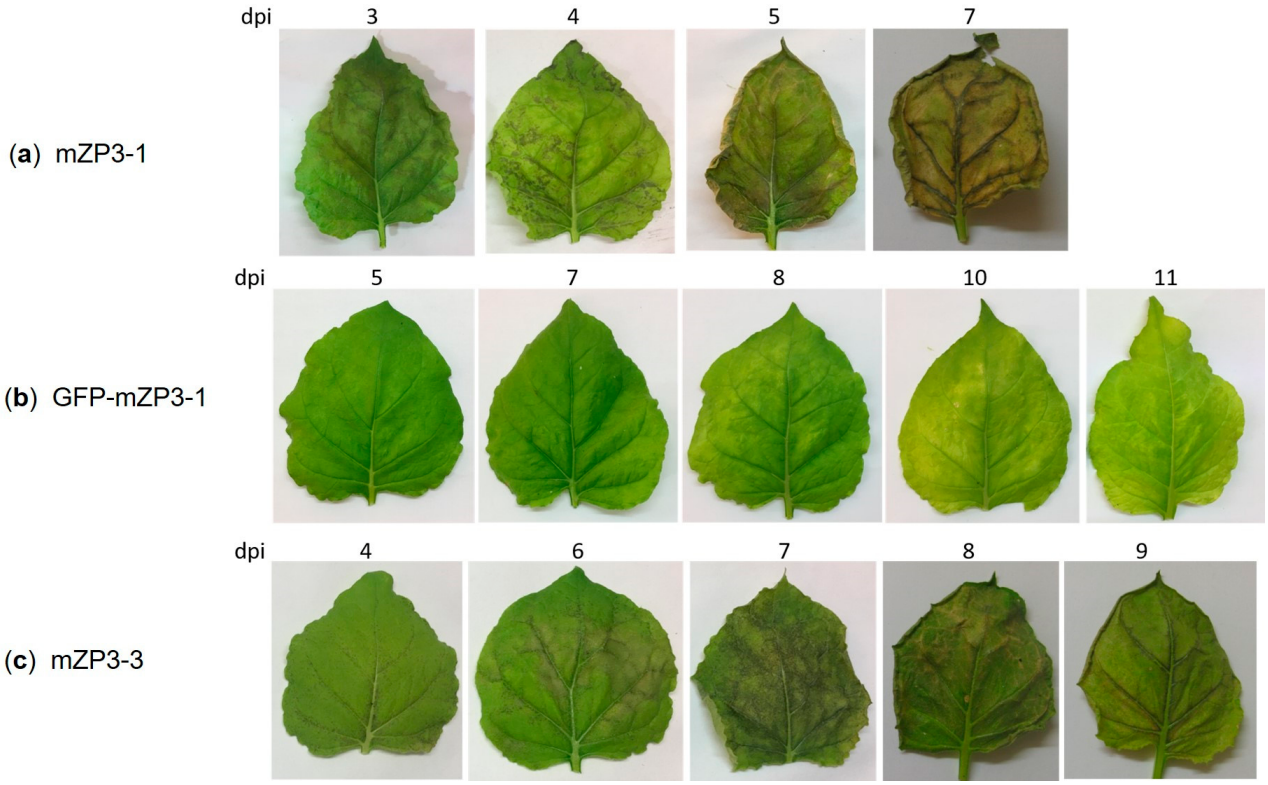

Figure 4. Morphological changes in *N. benthamiana* leaves agroinfiltrated with pICH31120 vector carrying mZP3-1 (**a**), GFP-mZP3-1 (**b**), or mZP3-3 (**c**) constructs. Protein expression induced chlorosis/necrosis and progressed to cell death on different days. Numbers represent days post-infiltration (dpi).

3.3. Purification and Characterization of Plant-Produced mZP3 Antigens

Purification of plant-produced mZP3-1, GFP-mZP3-1, and mZP3-3 proteins was carried out under non-denaturing conditions through Ni^{2+} -charged column chromatography. Fractions were collected and subjected to SDS–PAGE followed by western blots probed with mouse anti-His monoclonal antibodies. Although mZP3-1 was not detectable in crude protein extracts, analysis of the concentrated purified protein from the chromatography column revealed bands of proteins that confirmed recombinant expression of mZP3-1 (Figure 5). The 12 kDa band detected by western blot corresponds to the expected size of the glycosylated mZP3-1 protein; however, possible glycosylation could not be detected by lectin blotting. Bands with higher molecular mass were also observed in the western blot, which are close to the molecular mass of oligomer forms of the mZP3-1 protein. The infiltrated plant leaves showed the highest expression of mZP3-1 at day 4 post infiltration; however, the yield of mZP3-1 was low compared to that of the other two proteins. Figure 5 illustrates 30× concentrated purified mZP3-1 compared to 1× purified GFP-mZP3-1 and mZP3-3 proteins.

The concentration of plant-made mZP3 peptide in crude protein extract and in antigens purified from *N. benthamiana* infiltrated leaves was quantified with ELISA. Compared with mZP3-1, GFP-mZP3-1 and mZP3-3 increased the production of the mZP3 peptide by more than 20 and 25 times, respectively (Table 1). However, among the three purified proteins, the highest concentration of mZP3 peptide was obtained in GFP-mZP3-1 (53.4 ± 1 µg/g DW). The purified mZP3-1 and mZP3-3 revealed 3.4 ± 0.8 µg/g DW and 32.7 ± 2.3 µg/g DW mZP3 peptide, respectively (Table 1). The accumulation of mZP3-1 was not sufficient for application in mouse vaccination. Therefore, only GFP-mZP3-1 and mZP3-3 were purified

on a large scale from *N. benthamiana* leaves at the optimal harvest time and prepared for vaccination of female BALB/c mice. Ni-NTA affinity purified proteins were concentrated and desalted using Vivaspin™ ultrafiltration spin columns. Impurities were removed from samples using a 0.22 µm syringe filter.

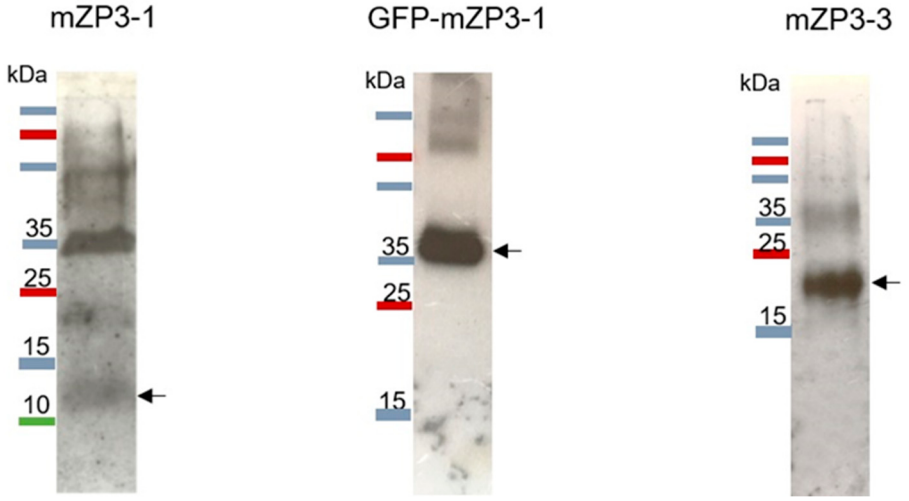

Figure 5. Detection of purified plant-expressed recombinant proteins using Ni-NTA affinity chromatography. Western blot of the 30× concentrated elution fraction from mZP3-1 in comparison to the unconcentrated elution fraction from GFP-mZP3-1 and mZP3-3. Arrows indicate the bands at the expected size of the glycosylated proteins. Western blots probed with mouse anti-His monoclonal antibodies. Blots cropped for clarity, for uncropped blots see Figure S5. For Coomassie-stained SDS-PAGE of purified plant-expressed recombinant proteins, see Figure S6.

Table 1. mZP3 accumulation levels in crude plant extract and in purified antigens, as determined using ELISA.

Antigen	Time Point	mZP3 Peptide/TSP (%)	Purified mZP3 (µg/g LDW)
mZP3-1	4 dpi	0.047 ± 0.006	3.4 ± 0.8
GFP-mZP3-1	8 dpi	0.95 ± 0.05	53.4 ± 1
mZP3-3	6 dpi	1.18 ± 0.12	32.7 ± 2.3

Infiltrated *N. benthamiana* leaves were collected at the optimal time point and evaluated for mZP3 expression by ELISA. Data are reported as the mean ± SEM from three independent infiltrated samples. dpi, days post-infiltration; TSP, total soluble protein; LDW, leaf dry weight.

3.4. Immunogenicity of mZP3-Based Antigens

To determine whether the plant-produced mZP3-based antigens are immunogenic in mice, a prime-boost immunization assay was conducted (Figure 6a). Two groups of five female BALB/c mice were immunized and boosted two times at three-week intervals with either recombinant GFP-mZP3-1 or mZP3-3 containing 50 µg mZP3 peptide. Additionally, the third group of five mice received PBS as a control. The antibody responses of immunized mice were assessed using ELISA. Figure 6b illustrates the mean endpoint titer of IgG antibodies in pre-immune sera and sera of the immunized mice after each immunization. Although an immune response was not observed after prime injection in GFP-mZP3-1-immunized mice, serum antibody responses increased greatly after the first booster and showed a significant difference compared to the control group. All GFP-mZP3-1-immunized mice produced detectable IgG antibodies at serum dilutions up to 1:8000 (log2, 12.96) after the first boost dose. Antibody levels continued to increase in all vaccinated mice following the second boost dose. However, there were no detectable IgG antibody titers in all sera of control mice throughout the whole course (Figure 6b).

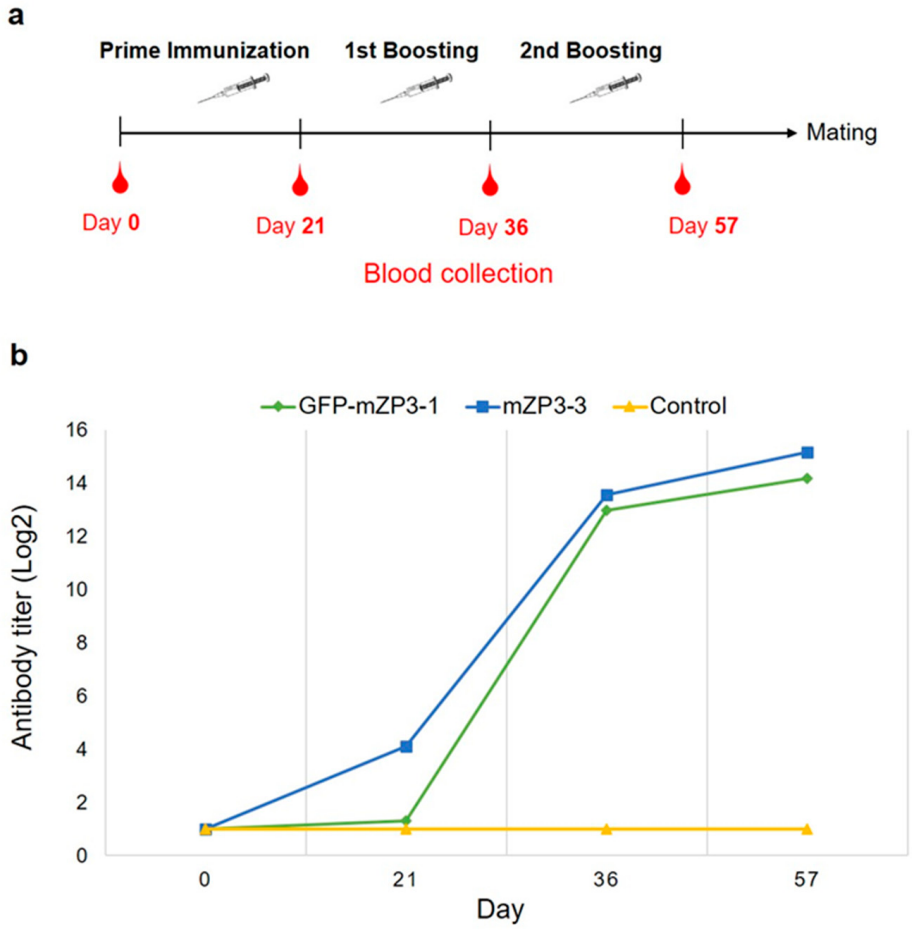

Figure 6. Kinetics of serum antibody responses in female BALB/c mice following prime-boost parenteral immunization. The mice were separated into three groups; two experimental groups were immunized with 50 µg of ZP3 peptide, in the form of GFP-mZP3-1 or mZP3-3, along with Polygen adjuvant, and the Control group was immunized with PBS adjuvanted by Polygen. (**a**) Schematic representation of the immunization protocol and blood collection. (**b**) The endpoint titers of anti-mZP3 antibodies in BALB/c mice immunized with plant-produced GFP-mZP3-1 (♦), mZP3-3 (■), or PBS as a control (▲) on days 0, 21, 36, and 57. The values are the means log2 endpoint titers of all mice in each group (N = 5).

In mice immunized with plant-produced mZP3-3, only one mouse produced detectable IgG antibodies at a 1:4000 dilution after the priming dose. However, antibody titers increased drastically in the remaining animals after the first boost dose. Although there was variation between the IgG titers of individuals, antibody responses were detectable in all mZP3-3-immunized animals at serum dilutions higher than 1:8000. The antibody titer subsequently increased in all mice following the second booster (Figure 6b). Two mice showed antibody responses even up to a titer of 1:64,000 (log2, 15.96) after the second boost injection.

These results indicated that both plant-produced antigens were capable of inducing antibodies against mZP3, which increased following each injection and reached the peak after the final boost dose. Figure 7 represents the comparison of antibody responses in mice from the three groups after the final boost injection at a 1:1000 dilution. The results showed a significant difference not only between the experimental groups and control ($p < 0.001$) but also between the experimental groups (Figure 7). Mice immunized with the plant-produced mZP3-3 antigen elicited a significantly higher antibody response ($p < 0.05$) than those immunized with the GFP-mZP3-1 antigen after the second booster.

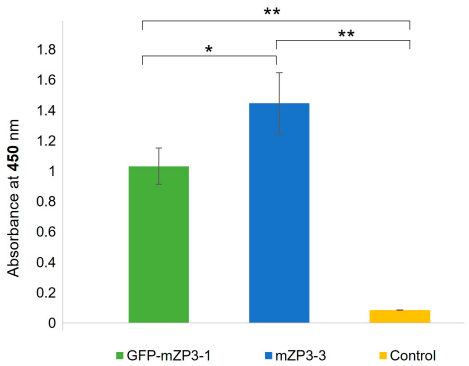

Figure 7. Comparison of serum antibody responses in mice immunized with GFP-mZP3-1, mZP3-3, or PBS after the final boost injection. Absorbance values are shown for sera of each immunized group at a 1:1000 dilution. Data are presented as the mean ± SD absorbance values at 450 nm. * $p < 0.05$; ** $p < 0.001$.

3.5. Fertility and Ovarian Effects in BALB/c Mice Immunized with mZP3 Antigens

To assess the fertility of female mice immunized with GFP-ZP3-1 or mZP3-3 antigens or PBS, individuals were mated with BALB/c males of similar age three weeks after the final immunization. Female mice were allowed to produce litters. The results showed that there was no significant reduction in the fertility or litter size of mice immunized with plant-produced GFP-ZP3-1 or mZP3-3 antigens. All animals in the treated groups and control group were fertile. No significant reduction in the mean litter size of mice immunized with recombinant GFP-mZP3-1 was achieved (Table 2). Although one animal from the mZP3-3 group showed a reduction in litter size, the mean litter size of this group was not significantly different from that of the control group (Table 2). The mean litter sizes of mice immunized with GFP-mZP3-1 (5.7 ± 0.6) and mZP3-3 (7 ± 1.5) were not significantly different from those of control mice (7 ± 0.9).

Table 2. Fertility of BALB/c mice immunized with recombinant mZP3-based antigens.

Antigen	Total Mice	Number of Injection	Number of Fertile Mice	Litter Size (Mean ± SEM)	*p*
GFP-mZP3-1	5	3	5/5	5.7 ± 0.6	N/S
mZP3-3	5	3	5/5	7 ± 1.5	N/S
PBS	5	3	5/5	7 ± 0.9	

Female BALB/c mice were immunized and boosted two times at three-week intervals with antigens in Polygen adjuvant and mated three weeks after the final boost. Values are means ± SEM. The *p* value indicates a significant difference ($p < 0.05$) between the treated and control groups. N/S, not significant.

Histological examination of the ovaries of immunized mice showed no evidence of oophoritis or disrupted folliculogenesis. The ovaries demonstrated normal follicles at different stages of development (Figure 8).

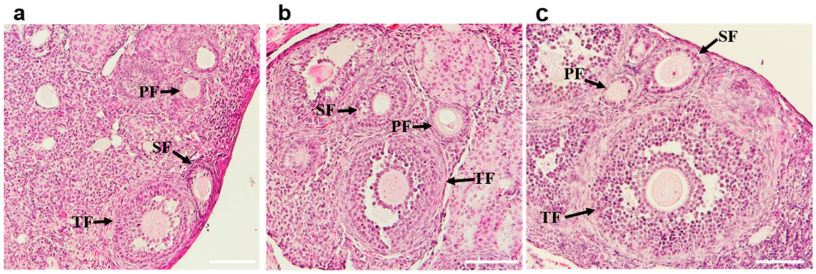

Figure 8. Histological analysis of the hematoxylin and eosin-stained ovarian sections of BALB/c mice. Ovaries of the mice immunized with GFP-mZP3-1 (**a**) and mZP3-3 (**b**) showing normal follicles at different stages of development as in ovaries from mouse immunized with PBS (**c**). PF, primary follicles; SF, secondary follicles; TF, tertiary (antral) follicles. Scale bars = 100 µm.

3.6. Reactivity of Serum Antibodies with the Mouse ZP Matrix by Indirect Immunofluorescence

An indirect immunofluorescence assay was conducted to evaluate the binding of serum antibodies to the ZP matrix in BALB/c and wild mice. The results showed that the antibodies generated against the plant-produced GFP-mZP3-1 or mZP3-3 did not react with the ZP matrix surrounding the oocytes in BALB/c mice. Fluorescence signals from immune sera were similar to those of pre-immune serum samples (Figure 9a). However, immunofluorescence studies with wild mouse ovarian sections showed that serum antibodies from both immunized groups recognized the ZP matrix. Immune sera of immunized mice bound to the ZP of wild mouse ovarian sections, whereas pre-immune serum samples from the same animal did not show reactivity to the ZP matrix (Figure 9b).

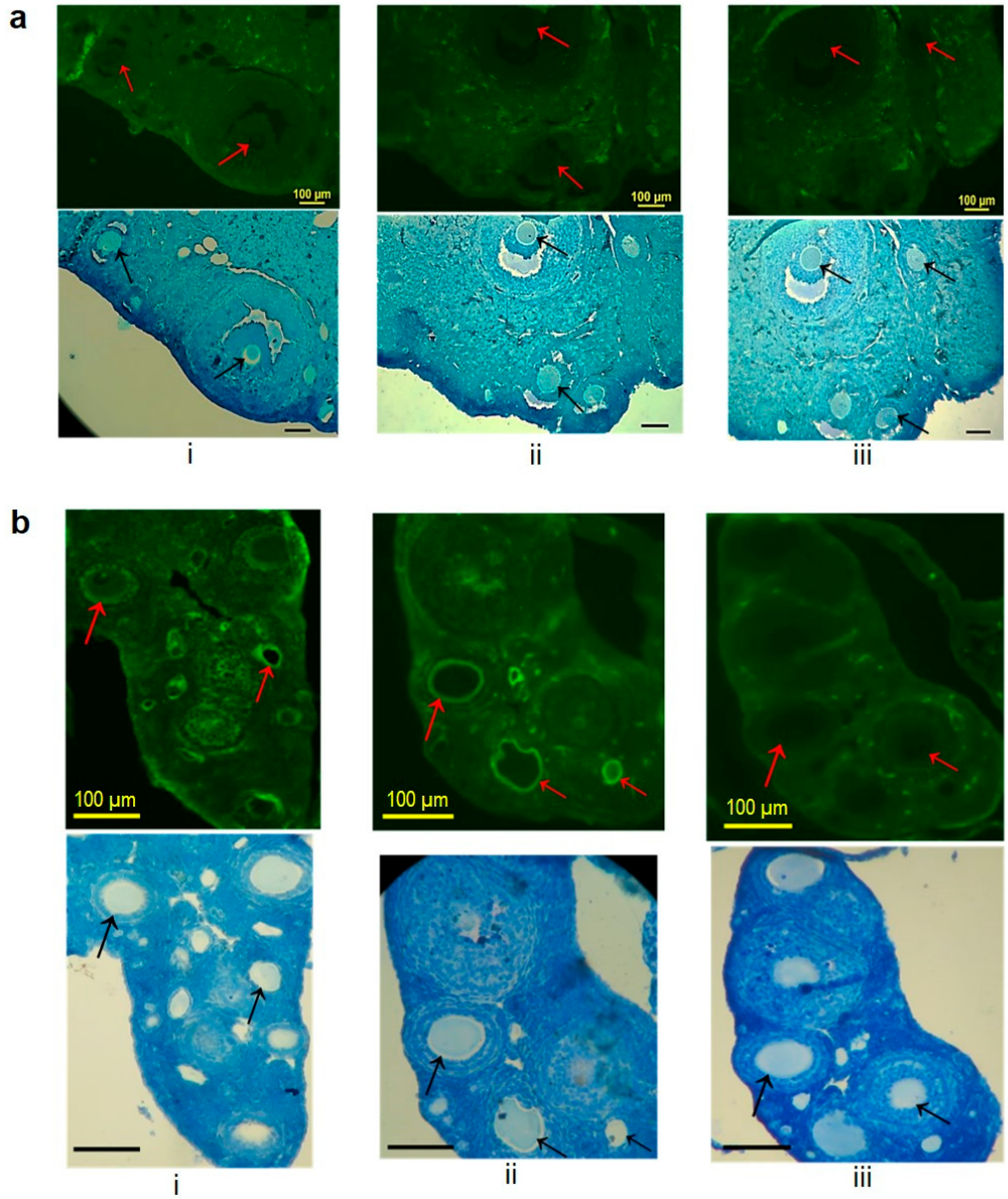

Figure 9. Indirect immunofluorescence microscopy for detecting the reactivity of serum antibodies with zona pellucida matrix in ovarian sections. Ovarian sections from BALB/c mouse (**a**) and wild mouse (**b**) treated with sera from mice immunized with GFP-mZP3-1 (i) and mZP3-3 (ii). Control experiments were performed with pre-immune sera of mZP3-3-immunized mice (iii). The upper pictures show sections in the fluorescent field, and the lower pictures represent stained sections in the bright field. Arrows represent the zona pellucida. Scale bars = 100 µm.

4. Discussion

In the face of an overpopulation of mice, developing a species-specific, inexpensive, effective, and readily applicable contraceptive vaccine is required. This study aimed to develop an immunocontraceptive vaccine based on the mZP3 glycopeptide in plants.

To develop contraceptive vaccines targeting wild mouse populations, an effective mouse-specific antigen is needed. Vaccines based on the mZP3 peptide appear to be promising since the peptide has been found to be sufficiently immunogenic and contraceptive in wild mice [43]. In addition, it is hypothesized to be mouse-specific because it has not been detected in other species such as hamster, guinea pig, cat, or dog [41,44]. Therefore, it might be a good choice for producing a mouse-specific contraceptive vaccine in transgenic plants that is a suitable platform for the rapid, low-cost, and large-scale production of vaccines [56,65]. Moreover, transgenic plants can facilitate the administration of vaccines by providing a potential platform for the oral delivery of vaccines (52), which could be an ideal method for vaccination of wildlife populations.

To create an effective vaccine, it is necessary to produce a sufficient amount of stable antigen. Our results indicated that mZP3-1 was unstable, with accumulation levels dropping four days after infiltration, before the optimal day proposed for the expression system [60,66]. The low mZP3-1 yield might be related to its susceptibility to proteolytic processes in the heterologous environment [58,67,68]. Fusing recombinant peptides or proteins with a partner that is stably expressed in plants is one approach to increase the yield of target proteins in plants [69]. Fusion of mZP3-1 to the GFP coding region, resulting in GFP-mZP3-1, led to a more than 25-fold higher accumulation of mZP3. GFP has already been used successfully to improve protein production and stability under various conditions [70–72]. This stabilization seems to depend not only on the GFP sequence, but also on the protein size since mZP3-3 showed a similar effect on the stability and accumulation of the protein.

The low amounts of mZP3-1 could also be due to the massive damage that mZP3-1 production caused in the plants. It has already been observed that transient expression of ER-targeted proteins can put stress on plants and cause prohibitive levels of tissue necrosis that consequently lead to low yields of recombinant proteins [73–75]. Expression of GFP-mZP3-1 and mZP3-3 reduced the severity of tissue necrosis and resulted in higher protein accumulation. Other studies have achieved an increase in target protein accumulation by reducing the ER stress response and moderating plant tissue necrosis [73,76]. Hence, differences in protein accumulation might be due not only to differences in protein stability, but also to differences in the induction of plant stress responses.

The other necessity for an efficient vaccine is sufficient immunogenicity. Immunization of mice with mZP3-3 antigens led to the generation of significantly higher antibody responses against mZP3 compared to GFP-mZP3-1. In line with this, previous studies have shown that antigens containing multiple repeats of ZP3 epitopes stimulate higher antibodies in BALB/c mice compared with those containing single mZP3 epitopes [32,77]. Incorporation of multiple copies of epitopes into antigens has been investigated previously for various weak immunogens to enhance their immunogenicity [78–80].

Notably, the production of mZP3 antigens in plants seems to increase immunogenicity compared to other production systems. *E. coli*-produced single mZP3-peptide antigens elicited no significant antibody responses to ZP3 in BALB/c immunized mice [32], and ZP3 protein expressed in the insect cell-expression system [81] seems to induce lower levels of antibodies against mZP3 peptide compared to our study. These observations highlight the importance of the expression system for the production of recombinant ZP3 antigens and show the superiority of plants over other expression platforms in this case. The importance of glycosylation in the efficiency of recombinant mouse ZP3 antigens as immunocontraceptive has been already demonstrated [81]. Plants offer the ability to perform posttranslational modification of proteins in a similar, but not equal, way compared to mammalian proteins that can support the immunogenicity of plant-made vaccines [51,67,82]. Although glycosylation analysis was not carried out, the strong reaction

of plant-produced proteins with the carbohydrate-binding protein Con A and the size of the proteins may be evidence for the glycosylation of the mZP3 peptide. Therefore, oligosaccharide residues present in plant-produced mZP3 may be a contributing factor in increasing the immunogenicity of antigens in vaccinated mice. Nevertheless, it might also indicate the stimulating effects of the TT epitope as an adjuvant. It has been observed previously that the antibody responses to ZP3 improved when additional appropriate T-cell helper epitopes were included in antigens [10,83].

Wild mice cause huge problems in immunization and mating studies due to their aggressive behavior. Hence, we had to restrict studies with live animals to the laboratory BALB/c mice used in most previous studies. Accordingly, despite high anti-mZP3 antibody titers, reproduction rates were not significantly reduced in immunized mice. It has been suggested that contraception depends on additional factors, such as mouse genetic background in combination with immune responses [32,43]. However, mZP3 peptide has been effective in reducing fertility in wild mice [43]. Moreover, the infertility of mice correlated with the reaction of antibodies to the zona pellucida. Hence, the immunological cross-reactivity between serum antibodies and wild mouse ZP observed in our study may reflect the ability of anti-mZP3 antibodies to bind to the ZP *in vivo* in wild mice. Therefore, further research is required to evaluate the contraceptive effect of plant-made mZP3 in wild mice.

In conclusion, the present study demonstrates that mZP3 can be effectively produced in *N. benthamiana* plants using a transient expression system. Compared to other production systems, plants seem to be more promising for producing highly immunogenic mZP3 antigens. Among the antigens studied, mZP3-3 is the best choice because it was efficiently expressed in the plant and induced high levels of antibodies that can react to the native zona pellucida of wild mice as the target species. The study is limited by the lack of information on the effect of plant-produced mZP3 in reducing the fertility of wild mice. Therefore, further research will be conducted to determine the contraceptive efficiency of plant-made mZP3-based antigens in wild mice. Furthermore, the practicality of injectable vaccines for wild pest animals is limited, so there is a need to formulate the plant-made mZP3 as effective bait for oral vaccination of mice.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/vaccines11010153/s1. The following information is available. Figure S1: Protein and nucleotide sequences codon-optimized for *N. benthamiana*. Figure S2: Western blot of crude leaf extracts of *N. benthamiana* leaves infiltrated with mZP3-1. Figure S3: Western analysis of plant-produced GFP-mZP3-1 and mZP3-3. Figure S4: Profile of lectin binding to the *N. benthamiana*-expressed proteins. Figure S5: Detection of purified plant-expressed recombinant proteins. Figure S6: Coomassie-stained SDS-PAGE of purified plant-expressed recombinant proteins.

Author Contributions: K.G. designed and performed the experiments, analyzed the data, and wrote the manuscript. I.B. and J.H. supervised the work, gave scientific advice, and revised the manuscript. J.S. supervised the histological and immunofluorescence studies. N.K. and R.K. performed the animal experiment. All authors approved the manuscript. All authors have read and agreed to the published version of the manuscript.

Funding: The plant work was funded by the scholarship of the 'Landesgraduiertenförderung Mecklenburg-Vorpommern' of the University of Rostock.

Institutional Review Board Statement: The animal study was approved by Landesamt fur Landwirtschaft, Lebensmittelsicherheit und Fischerei, Mecklenburg-Vorpommern, Germany (Protocol number 7221.3-1-073/17 and 7221.3-3.2-002/22).

Informed Consent Statement: Not applicable (the study did not involve humans).

Data Availability Statement: The datasets generated during this study are available from the corresponding author upon request.

Acknowledgments: The authors would like to acknowledge Udo Meyer and Susanne Montag, Bioserv GmbH Rostock, for animal experiments and the production of anti-mZP3 antibodies. We are grateful to Susanne Radloff and Simone Krüger—from the Department of Life, Light & Matter, University of Rostock—for providing the fluorescence microscope and for their assistance. We are also grateful to Kerstin Thoss for technical assistance with the ELISAs.

Conflicts of Interest: The authors declare no conflict of interest related to the contents of this article.

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Supplementary Material to 2.1

a


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 M  Q  Y  I  K  A  N  S  K  F  I  G  I  T  E  L  R  S  G  G
ATGCAATATATCAAGGCTAATTCCAAGTTCATAGGCATTACTGAACTTAGATCTGGTGGA
                                                  mZP3-T
 G  G  S  G  G  G  G  S  G  G  G  G  S  L  K  C  S  N  S  S
GGAGGATCTGGAGGTGGTGGGTCAGGTGGAGGTGGGTCACTTAAGTGTAGCAACAGTTCT
          mZP3-B
 S  S  Q  F  Q  I  H  G  P  R  C  T  H  H  H  H  H  H  S  E
AGTTCCCAATTTCAGATTCATGGCCCAAGATGTACACACCATCATCACCATCACTCTGAG

 K  D  E  L  *
AAAGATGAATTGTAA
```

b


```
 M  Q  Y  I  K  A  N  S  K  F  I  G  I  T  E  L  G  G  G  G
ATGCAATATATCAAGGCTAACTCTAAGTTTATCGGAATTACTGAACTTGGAGGAGGCGGG
                                              mZP3-T        mZP3-B
 S  G  G  G  G  S  G  G  G  G  S  C  S  N  S  S  S  S  Q  F
TCTGGTGGAGGAGGGAGTGGAGGAGGAGGGTCATGCTCTAATTCTTCCTCATCTCAGTTT

 Q  I  H  G  P  R  G  G  G  G  S  G  G  G  G  S  G  G  G  G
CAGATACATGGTCCGAGAGGAGGAGGCGGTTCAGGCGGAGGTGGTAGTGGTGGTGGTGGG
          mZP3-T        mZP3-B
 S  C  S  N  S  S  S  S  Q  F  Q  I  H  G  P  R  G  G  G  G
TCTTGTAGTAATTCCAGCTCTTCTCAATTCCAAATTCATGGTCCACGTGGTGGCGGAGGC
                                              mZP3-T        mZP3-B
 S  G  G  G  G  S  G  G  G  G  S  C  S  N  S  S  S  S  Q  F
TCAGGTGGTGGTGGCTCAGGAGGTGGCGGGTCTTGTTCCAATAGCAGTAGTTCACAATTC

 Q  I  H  G  P  R  H  H  H  H  H  H  S  E  K  D  E  L  *
CAGATTCATGGGCCTAGGCATCATCACCACCATCACAGCGAGAAAGATGAATTGTAA
```

Figure 2.1 S1: Protein and nucleotide sequences codon-optimized for *N. benthamiana*. The nucleotide and amino acid sequences of synthetized mZP3-1 (**a**) and mZP3-3 constructs (**b**). Nucleotides are represented in black and amino acids in blue. Mouse Zona pellucida 3-peptide (mZP3) sequences are underlined. The locations of the mouse ZP3 contraceptive T and B cell epitopes (mZP3-T and mZP3-B) are shown above the sequences.

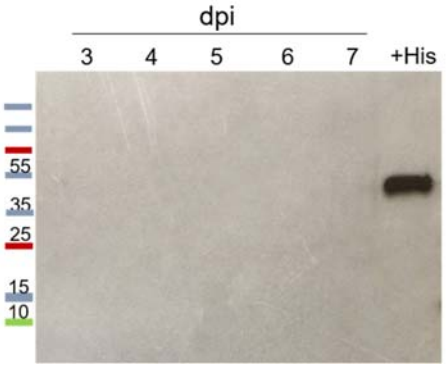

Figure 2.1 S2: Western blot of crude leaf extracts of *N. benthamiana* leaves infiltrated with mZP3-1. No protein band corresponding to the expected size of the mZP3-1 protein was detected. Western blot was probed with anti-His monoclonal antibody. +His, a His-tagged protein as a positive western blot control. dpi, days post infiltration.

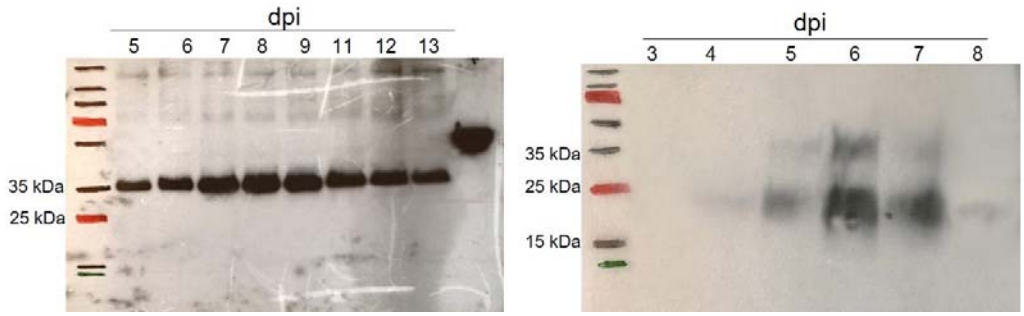

Figure 2.1 S3: Western analysis of plant-produced GFP-mZP3-1 (left) and mZP3-3 (right). Figure 2 was cropped from these western blot images. dpi, days post infiltration.

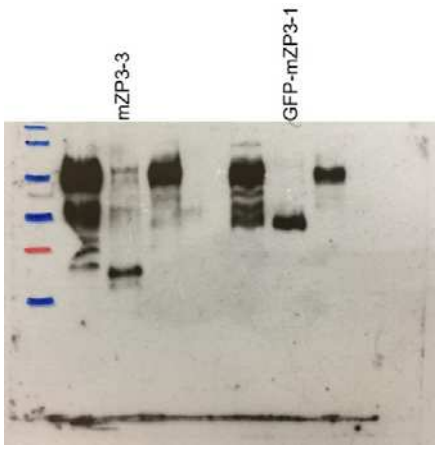

Figure 2.1 S4: Profile of lectin binding to the *N. benthamiana*-expressed proteins. The panels of figure 3 were cropped from this lectin blot image.

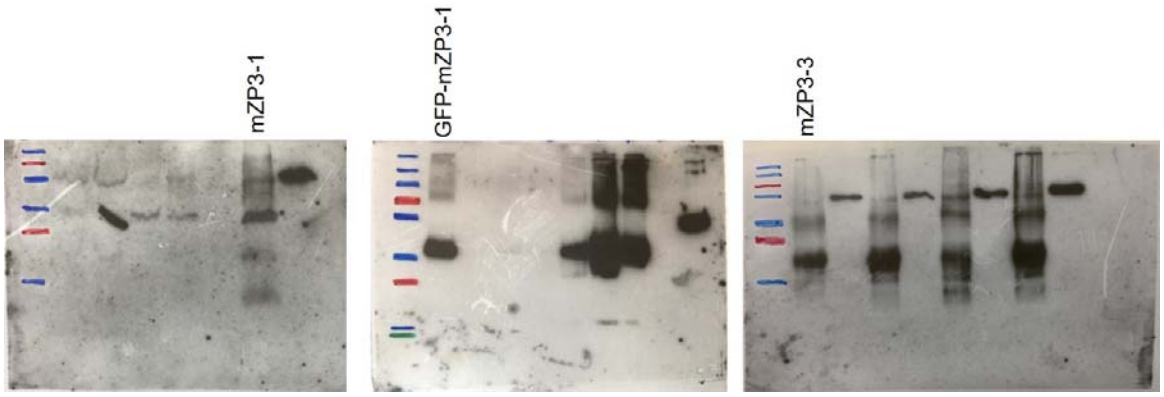

Figure 2.1 S5: Detection of purified plant-expressed recombinant proteins. The panels of figure 5 were cropped from these western blot images.

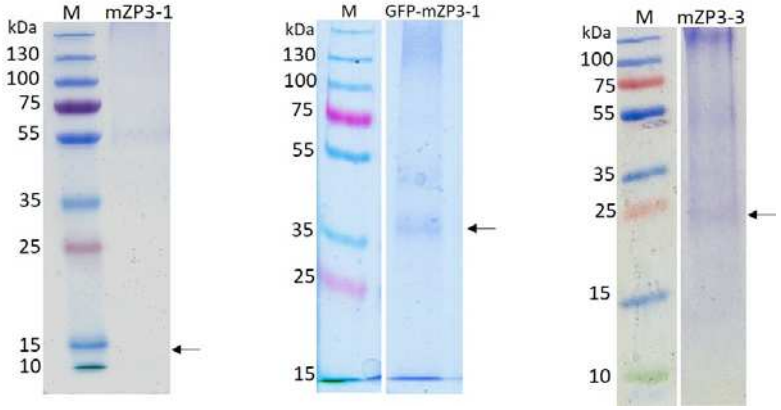

Figure 2.1 S6: Coomassie-stained SDS-PAGE of purified plant-expressed recombinant proteins. M, Marker. Arrows indicate the expected size of the proteins.

2.2 Oral and Subcutaneous Immunization with a Plant-Produced Mouse-Specific Zona Pellucida 3 Peptide Presented on Hepatitis B Core Antigen Virus-like Particles

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Published in

Vaccines (2023), 11(2), 462.

DOI: https://doi.org/10.3390/vaccines11020462

vaccines

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Oral and Subcutaneous Immunization with a Plant-Produced Mouse-Specific Zona Pellucida 3 Peptide Presented on Hepatitis B Core Antigen Virus-like Particles

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Abstract: A short mouse-specific peptide from zona pellucida 3 (mZP3, amino acids 328–342) has been shown to be associated with antibody-mediated contraception. In this study, we investigated the production of mZP3 in the plant, as an orally applicable host, and examined the immunogenicity of this small peptide in the BALB/c mouse model. The mZP3 peptide was inserted into the major immunodominant region of the hepatitis B core antigen and was produced in *Nicotiana benthamiana* plants via *Agrobacterium*-mediated transient expression. Soluble HBcAg-mZP3 accumulated at levels up to 2.63 mg/g leaf dry weight (LDW) containing ~172 µg/mg LDW mZP3 peptide. Sucrose gradient analysis and electron microscopy indicated the assembly of the HBcAg-mZP3 virus-like particles (VLPs) in the soluble protein fraction. Subcutaneously administered mZP3 peptide displayed on HBcAg VLPs was immunogenic in BALB/c mice at a relatively low dosage (5.5 µg mZP3 per dose) and led to the generation of mZP3-specific antibodies that bound to the native zona pellucida of wild mice. Oral delivery of dried leaves expressing HBcAg-mZP3 also elicited mZP3-specific serum IgG and mucosal IgA that cross-reacted with the zona pellucida of wild mice. According to these results, it is worthwhile to investigate the efficiency of plants producing HBcAg-mZP3 VLPs as immunogenic edible baits in reducing the fertility of wild mice through inducing antibodies that cross-react to the zona pellucida.

Keywords: contraceptive vaccine; zona pellucida 3 peptide; chimeric virus-like particles; plant molecular farming; oral vaccine; *Nicotiana benthamiana*

Citation: Ghasemian, K.; Broer, I.; Schön, J.; Killisch, R.; Kolp, N.; Springer, A.; Huckauf, J. Oral and Subcutaneous Immunization with a Plant-Produced Mouse-Specific Zona Pellucida 3 Peptide Presented on Hepatitis B Core Antigen Virus-like Particles. *Vaccines* **2023**, *11*, 462. https://doi.org/10.3390/vaccines11020462

Academic Editors: Esther Blanco and Juan Bárcena

Received: 20 January 2023
Revised: 14 February 2023
Accepted: 16 February 2023
Published: 17 February 2023

1. Introduction

Rodents cause economic, social, and environmental damage worldwide and can endanger the health of humans, companion animals, and livestock [1,2]. They can be one of the serious threats to food production in many parts of the world, such as Europe [3], Africa [4,5], Australia [6], and Asia [7,8]. It is necessary to manage the population of rodent pests with a species-specific and more humane approach to reduce their abundance below the damage threshold [9]. Fertility control has become an acceptable method of wildlife population management compared to conventional lethal methods such as culling, trapping, and poisoning that can threaten non-target species and the environment [10–12]. Immunocontraception, the prevention of conception through immunological methods, has been considered a potentially useful method, especially in multiparous species such as mice. Immunocontraception based on gamete-specific molecules involved in sperm–egg

interactions is more suitable for achieving acceptable and efficient contraception [13–15]. The zona pellucida (ZP) is an extracellular glycoprotein matrix that surrounds growing mammalian oocytes and ovulated eggs and is an essential molecule in sperm–egg interaction and fertilization [13,16,17]. Immunization against mouse ZP proteins has been extensively investigated, and the potential of ZP as a target to limit mouse populations has been demonstrated [11,18,19].

Murine ZP is composed of three glycoproteins: ZP1, ZP2, and ZP3. Mouse ZP3, the primary sperm receptor and acrosome reaction inducer, plays a key role in fertilization and has been suggested as an immunocontraceptive antigen [20,21]. The essential role of the ZP3 glycoprotein in fertilization has been substantiated using ZP3-knockout female mice; ZP3-null female mice are completely infertile [22,23]. Recombinant murine ZP3 antigens have been shown to induce effective contraceptive responses in immunized mice [12,19,24]. Nevertheless, contraceptive vaccines should be effective, inexpensive, and readily applicable. An additional crucial requirement, especially for the field application of contraceptive vaccines, is the species-specificity of the vaccine, which cannot be addressed using protein-based antigens [13,17].

The development of peptide contraceptive antigens can improve the safety and specificity of vaccines [25], thereby allowing the oral application of vaccines for wildlife population control. Peptide-based contraceptive vaccines that target species-specific and functional regions of reproductive antigens can increase the target specificity and reduce the risk of side effects [26,27]. Amino acid residues on ZP3, which are poorly conserved between species, have been described to be involved in sperm binding, and antibodies against these amino acids interfere with sperm binding [28–30]. A mouse-specific ZP3 glycopeptide (mZP3), corresponding to amino acid residues 328–342 that contain epitopes (B- and T-cell epitopes) associated with antibody-mediated contraception, has been shown to induce contraceptive antibodies in different mouse strains, including wild mice [31–33]. Nevertheless, the immunogenicity of small peptide-based vaccines results in reduced efficacy [25,34]. To compensate for this, an immunogenic carrier molecule can be used to improve the immunogenicity of vaccines [35,36].

One method that has proven successful in carrying and delivering peptide epitopes is the use of virus-like particles (VLPs), which are non-infectious particles assembled from viral structural proteins [37]. In this study, the hepatitis B core antigen (HBcAg) was used to present the contraceptive mZP3 peptide. Several studies have described the strong immunogenicity of HBcAg VLPs [38–40]. HBcAg VLPs are promising carriers for enhancing vaccine immunogenicity [41]. The hepatitis B core (HBc) particles consist of 180 or 240 HBcAg monomers that form dimeric units. The dimers are able to self-assemble into two types of icosahedral particles, small or large, with T = 3 (90 dimers) or T = 4 (120 dimers) symmetry, respectively. The repetitive structure and the presence of immunogenic epitopes within HBcAg confer superior properties and make HBcAg a very strong immunogen [36,37,42]. Numerous foreign epitope sequences can be introduced into different regions of HBcAg without interfering with the VLP assembly. The major immunodominant region (MIR) or c/e1 loop of the core protein, located at the tip of spikes on the surface of assembled particles, is the most favorable site for epitope presentation [43,44]. Epitopes inserted at this site can be repetitively presented on the surface of particles; multimeric presentation of the displayed epitopes leads to a high level of immunogenicity [42,45]. Thus, the mZP3 peptide sequence was incorporated into the MIR to benefit from the immune-enhancing properties of the HBcAg VLPs.

Although the delivery of contraceptives by traditional methods (dart or manual injection) may be suitable for larger mammals, oral vaccine delivery is more promising for population management and control of small animals, such as mice, which are dispersed over a large area. Plants are considered to have the potential for the production and oral delivery of vaccines, which can be directly used as feed and eliminate the purification or processing of antigens [46,47]. Plant-made vaccines can be protected from degradation in the stomach through bioencapsulation in plant cells [48,49] and can induce systemic (IgG)

and mucosal (IgA) immunity [50,51]. Plants offer advantages, such as scalability, safety, ease of storage, cost efficiency, and eukaryotic protein processing [52,53]. Moreover, since mZP3 is naturally glycosylated and glycosylation can affect the efficiency of recombinant ZP3-based antigens [24], plants could be a suitable system for the expression of glycosylated mZP3. *Agrobacterium*-mediated transient transformation of plants provides a method for rapid and large-scale production of recombinant proteins in plants [54]. *Nicotiana benthamiana* is one of the most efficient plant hosts and is well studied and commonly used to produce high levels of transiently expressed proteins [55,56]. Using the MagnICON transient expression system, high yields of recombinant proteins (up to 80% of the total soluble protein) have been achieved within a few days in *N. benthamiana* [57,58].

In the present study, we aimed to produce HBcAg particles displaying the contraceptive mZP3 peptide in *N. benthamiana* using a MagnICON-based transient expression system, as a fast system for proof of concept. Because immunization experiments in wild mice are problematic, the immunogenicity of plant-produced HBcAg-mZP3 was investigated in BALB/c mice to produce anti-mZP3 antibodies, which were used in immunohistochemistry on ovarian sections from wild mice to assay antibody–oocyte interaction. Plant-produced HBcAg-mZP3 was used in the parenteral and oral immunization of female mice to evaluate the systemic and mucosal immunogenicity of HBcAg-mZP3 in immunized mice.

2. Materials and Methods

2.1. Construction of HBcAg-mZP3 Expression Vector

The full-length monomeric HBcAg gene was kindly provided by Dr. Hadrien Peyret (John Innes Center, UK). BsaI restriction sites were introduced at the 5′ and 3′ ends through amplification of the HBcAg gene, and the PCR fragment was cloned into the pJET1.2 cloning vector (CloneJET PCR Cloning Kit; Thermo Fisher Scientific, Braunschweig, Germany). The insert sequence, mZP3 peptide CSNSSSSQFQIHGPR corresponding to amino acid residues 328–342, was flanked by flexible GGGGS linkers. It was synthesized with codon usage optimized for expression in *N. benthamiana* (Eurofins MWG Operon, Ebersberg, Germany). The sequence was inserted into the MIR of HBcAg using SalI and AvrII restriction sites, which are the unique restriction sites at the c/e1 loop region of the HBcAg for ease of cloning. The resulting construct was named HBcAg-mZP3. To target HBcAg-mZP3 to the secretory pathway for facilitated glycosylation [59,60], the coding sequence of HBcAg-mZP3 was cloned into the TMV-based expression vector pICH31120 of the MagnICON system by BsaI restriction/ligation [61]. The MagnICON expression vectors were kindly provided by Nomad Bioscience GmbH (Halle/Saale, Germany). The identity of HBcAg-mZP3 was confirmed by sequencing.

2.2. Expression of HBcAg-mZP3 in N. benthamiana Leaves

The recombinant expression vector pICH-HBcAg-mZP3 was introduced into *Agrobacterium tumefaciens* strain ICF320 by electroporation. The resulting transformed colonies (confirmed by colony PCR and restriction enzyme digestion) were propagated in Luria–Bertani (LB) medium supplemented with 50 µg/mL kanamycin and 50 µg/mL rifampicin and incubated overnight in a shaking incubator (220 rpm, at 28 °C). To prepare the main bacterial culture, two milliliters of the starter culture was diluted in 200 mL LB, containing the same antibiotics, and incubated overnight under shaking conditions at 28 °C. The *agrobacterium* cells were sedimented at 4560× *g* for 30 min, and the pellet was resuspended in infiltration buffer containing 10 mM 2-(N-morpholino) ethanesulfonic acid (MES), pH 5.8, 10 mM $MgSO_4$, and 0.02% *v*/*v* Silwet Gold to a final OD_{600} of 0.15–0.2. The bacterial suspension was vacuum-agroinfiltrated into the leaves of 6–8 weeks old greenhouse-grown *N. benthamiana* plants as previously described [62].

2.3. Protein Extraction

Small-scale protein extraction was performed for protein expression and accumulation analysis. Infiltrated leaves were harvested on different days post-infiltration (dpi) and

frozen at −80 °C. The frozen samples were lyophilized for 72 h using an Alpha 1–4 LD plus freeze-dryer (Martin Christ GmbH, Osterode, Germany) and then ground to fine powder. Protein extraction was performed using four protein extraction buffers to optimize the extraction of HBcAg-mZP3 VLPs: extraction buffer 1 (50 mM sodium phosphate, pH 7.4, 100 mM NaCl, 50 mM Tris-HCl, 10 mM ethylenediaminetetraacetic acid (EDTA)), extraction buffer 2 (50 mM Tris-HCl, pH 7.25, 150 mM NaCl, 2 mM EDTA, 0.1% *w*/*v* Triton X-100, 1 mM dithiothreitol (DTT)), extraction buffer 3, PBS buffer (100 mM NaCl, 10 mM KCl, 6.5 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.2), and extraction buffer 4 (10 mM NaH_2PO_4-H2O, 300 mM NaCl, 25 mM Tris-HCl, pH 7.4) plus complete protease inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany). Dry leaf samples were homogenized in ice-cold extraction buffer using a Precellys 24 homogenizer (Bertin Instruments, France). The clarified extract was obtained by repeated centrifugation at 15,000× *g* for 15 min at 4 °C. The supernatant was recovered as the soluble (S) protein fraction. The insoluble protein (IS) fraction was extracted by boiling the pellet in 1X SDS sample buffer and centrifugation at 15,000× *g* for 15 min.

2.4. Purification of Virus-like Particles (VLPs)

For large-scale extraction, infiltrated leaves were harvested at 8 dpi, lyophilized, and pulverized as described above. Ground leaf material was homogenized in chilled extraction buffer 2 using a homogenizer (Polytron PT 2100, Kinematica AG, Switzerland) for 30 s at 30,000 rpm. Homogenized tissue was rotated at 4 °C for 30 min to increase solubility. The homogenate was centrifuged at 4600× *g* for 15 min, and the extract was clarified by repeated ultracentrifugation at 16,000× *g* for 30 min at 4 °C.

To purify the assembled particles, the clarified extract was subjected to sucrose gradient sedimentation as previously described [63]. Briefly, the plant extract was layered at the top of a 10–60% (*w*/*v*) sucrose density gradient (dissolved in 10 mM Tris-HCl pH 8.4, 120 mM NaCl) and centrifuged in a TH-641 swing-out rotor at 187,000× *g* for 2.5 h at 4 °C. The gradient was fractionated and analyzed by Western blotting. In the second approach, VLPs were purified using double-layered sucrose cushions as previously described [64]. Briefly, clarified protein extracts were loaded above 2 mL of 25% (*w*/*v*) and 0.3 mL of 70% (*w*/*v*) sucrose and centrifuged as above. After ultracentrifugation, eight fractions were recovered from the bottom of the tubes and assayed for HBcAg-mZP3 content and VLP assembly using Western blotting and electron microscopy.

2.5. SDS-PAGE and Western Blot Analysis

Samples containing HBcAg-mZP3 were subjected to 12% SDS-PAGE after denaturation at 95 °C for 7 min in loading buffer (10% glycerin, 150 mM Tris (pH 6.8), 3% SDS, 1% β-mercaptoethanol, and 2.5% bromophenol blue). The separated proteins were transferred to nitrocellulose membranes (GE Healthcare Europe GmbH, Solingen, Germany). Membranes were first blocked with 5% (*w*/*v*) non-fat milk powder in TBST (20 mM Tris, 150 mM NaCl, and 0.05% (*v*/*v*) Tween20, pH 7.6) for 2 h at 20–22 °C (room temperature (RT)). After three washes with TBST, the membranes were probed with rabbit anti-mZP3 primary antibody (Bioserv, Rostock, Germany) to detect the mZP3 component of the protein. Following another washing step, the membranes were further probed with horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibody (Dianova, Hamburg, Germany) diluted 1:2000 in blocking buffer for 1 h at RT. The signals were then developed using ECL chemiluminescence reagents and detected by exposing the membranes to Kodak Biomax light X-ray film (VWR; Darmstadt, Germany). Concanavalin A (Con A) lectin blot analysis was conducted as previously described [62], to visualize the putative glycosylation of plant-made mZP3.

2.6. ELISA

The expression pattern of HBcAg-mZP3 was examined by ELISA that detects the mZP3 peptide. Briefly, the plates were coated with plant protein extract diluted in carbonate buffer

(pH 9.6) for 2 h at RT. The plates were washed thrice with PBS containing 0.05% Tween 20 (PBST) and blocked further with 1X RotiBlock blocking solution (Carl Roth GmbH + Co. KG, Karlsruhe, Germany) for 1 h at RT. A rabbit anti-mZP3 antibody (Bioserv) was used as the primary antibody and incubated for 2 h at RT. Following another washing step, the plates were treated with HRP-conjugated goat anti-rabbit secondary antibody (Dianova) diluted 1:2000 in PBS. The plates were developed with a tetramethylbenzidine (TMB) substrate, the reaction was stopped with 250 mM H_2SO_4, and the absorbance (OD) was measured at 450 nm. To eliminate non-specific OD values, the extract of leaves infiltrated with pICH31120 was used as a control. *N. benthamiana*-produced mZP3 with defined quantity [65] was used as a reference standard. The amount of HBcAg-mZP3 protein was estimated based on the ratio of its molecular weight to the mZP3.

2.7. Transmission Electron Microscopy (TEM)

The partially purified HBcAg-mZP3 VLPs were fixed in fixative buffer (1% paraformaldehyde, 2% glutaraldehyde in 0.1 M phosphate buffer, pH 7.3) and then subjected to negative staining with 2% uranyl acetate. The prepared samples were analyzed using a transmission electron microscope (EM 902A, Zeiss, Oberkochen, Germany) equipped with a tungsten cathode. Representative areas of the samples were analyzed at 80 kV accelerating voltage. Imaging was performed using a CCD camera (CCD-Sensor THX 7888A, 14 µm × 14 µm pixel size, 1024 × 1024 pixels per mm2) (Proscan, Scheuring, Germany). Image processing was performed using iTEM software (Olympus Soft Imaging Solutions GmbH, Münster, Germany).

2.8. Mouse Immunization and Specific Serum IgG and Fecal IgA Measurement

Animal experiments were conducted in accordance with German animal protection regulations, with the approval of Landesamt für Landwirtschaft, Lebensmittelsicherheit und Fischerei, Mecklenburg-Vorpommern, Germany (File No. 7221.3–1-073/17 and 7221.3–1-071/20–2).

Female BALB/c mice were used for the immunization studies. For systemic immunization, five mice were injected subcutaneously with partially purified plant-produced HBcAg-mZP3 containing 5.5 µg mZP3, and the control group received PBS. Mice were immunized thrice at three-week intervals. All doses contained 10% Polygen (MVP Lab) as an adjuvant. Blood samples were collected prior to the primary injection, three weeks after the first immunization, and two weeks after the second or third immunization to obtain serum samples. Sera were diluted to $1{:}10^5$ and subjected to ELISA.

To determine the mucosal immunogenicity of plant-produced HBcAg-mZP3, two groups of mice (n = 10) were administered 120 mg of lyophilized leaf material suspended in 1 mL PBS by gavage. The treatment group received leaves expressing HBcAg-mZP3 (containing approximately 190 µg mZP3), and the control group received pICH31120-infiltrated leaves. Serum was collected prior to primary oral immunization and 20 days after each immunization, diluted 1:100, and subjected to ELISA. Feces were collected before the first mucosal immunization and seven days after each immunization and stored at −80 °C until being assayed for antibodies secreted by the intestinal mucosa. Fecal pellets were suspended in five volumes (w/v) of PBS extraction buffer (500 µL buffer per 100 mg fecal material), vortexed, and left at RT for 1 h. The feces were homogenized using a Precellys 24 homogenizer (Bertin Instruments). The extract was clarified by repeated centrifugation at 32,000× g for 10 min at 4 °C. Fecal extract was collected and subjected to ELISA.

The anti-mZP3 IgG and IgA responses in serum and fecal samples were detected using ELISA. *N. benthamiana*-produced mZP3 was used to coat the plates. Plates were incubated with 1.5 µg mZP3 in carbonate/bicarbonate coating buffer (pH 9.6) for 2 h at RT and then blocked with 1% (w/v) bovine serum albumin (BSA) in PBS for 1 h at RT. Subsequently, the plates were treated with serum samples or fecal extracts diluted in PBS for 2 h at RT. To measure endpoint antibody titers, serially diluted samples were added to the wells.

After washing, the plates were incubated with HRP-conjugated donkey anti-mouse IgG (Dianova) or IgA (SouthernBiotech, Birmingham, Alabama, USA) diluted 1:2000 in PBS for 1 h at RT. After color development in the presence of TMB, the absorbance was measured at 450 nm. In the subcutaneous IgG assay, ELISA data were normalized by subtracting the non-specific readings (cells coated with extract of pICH31120-infiltrated leaves).

2.9. Indirect Immunofluorescence

Mouse ovarian sections were obtained from BIOSERV Analytik (Rostock, Germany). The sections of a wild mouse were deparaffinized and subjected to antigen retrieval using sodium citrate buffer (10 mM sodium citrate, 0.05% Tween 20, pH 6.0). Sections were blocked with 10% goat serum blocking solution (Life Technologies, Frederick, Maryland, USA) at RT for 1 h, washed with PBS, and incubated in 1X mouse-on-mouse IgG blocking solution (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) at RT for 1 h. Sections were incubated with a 1:20 dilution of serum samples or fecal extracts and kept at 4 °C overnight. The sections were washed and incubated with 10 µg/mL fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Invitrogen, Thermo Fisher Scientific) as the secondary antibody for 1.5 h at 37 °C. After washing, the slides were mounted with DABCO mounting medium (25 mg/mL DABCO, 90% glycerol, and 10% PBS, pH 8.5) and observed under a fluorescence microscope.

2.10. Statistical Analysis

The experimental data were analyzed using the IBM SPSS statistical software version 27. Means were compared using one-way analysis of variance (ANOVA). Duncan's test was used as a post hoc test to measure specific differences between the means. Statistical significance was set at $p < 0.05$.

3. Results

3.1. Design and Cloning of the HBcAg-mZP3 Expression Vector

According to Peyret (2015), the coding sequence of the mZP3 contraceptive peptide was inserted into the c/e1 loop region of the HBcAg. The mZP3 antigenic peptide was linked to HBcAg via GGGGS flexible linkers on either side, predicted to not restrict particle assembly. The HBcAg-mZP3 construct was cloned into the MagnICON-based plant expression vector pICH31120 to target HBcAg-mZP3 to the secretory pathway (Figure 1). *Nicotiana benthamiana* plants were infiltrated with *Agrobacterium tumefaciens* containing HBcAg-mZP3.

3.2. Transient Expression of HBcAg-mZP3 in N. benthamiana Plants

Following agroinfiltration, infiltrated leaves were harvested at 8 dpi, and HBcAg-mZP3 protein expression was investigated by Western blotting. Analysis of total soluble protein (TSP) extracted in four extraction buffers (EB) detected a protein band at the expected size of HBcAg-mZP3 using anti-mZP3 antibodies that specifically recognize mZP3. The results showed a monomeric form of the protein with a size of ~27 kDa and a higher protein band representing the dimer form of the protein. This protein was detected in leaves infiltrated with HBcAg-mZP3, whereas no protein band was found in extracts of leaves infiltrated with pICH31120, indicating the specificity of the HBcAg-mZP3 bands (Figure 2A). Analysis of equal amounts (30 µg) of TSP extracts showed that the highest amount of HBcAg-mZP3 was released into the TSP under EB2 conditions. However, a comparison between equal volumes of soluble and insoluble fractions from the EB2 condition showed that the majority of recombinant HBcAg-mZP3 remained insoluble (Figure 2B). More than 90% of the plant-produced HBcAg-mZP3 protein was estimated to be insoluble.

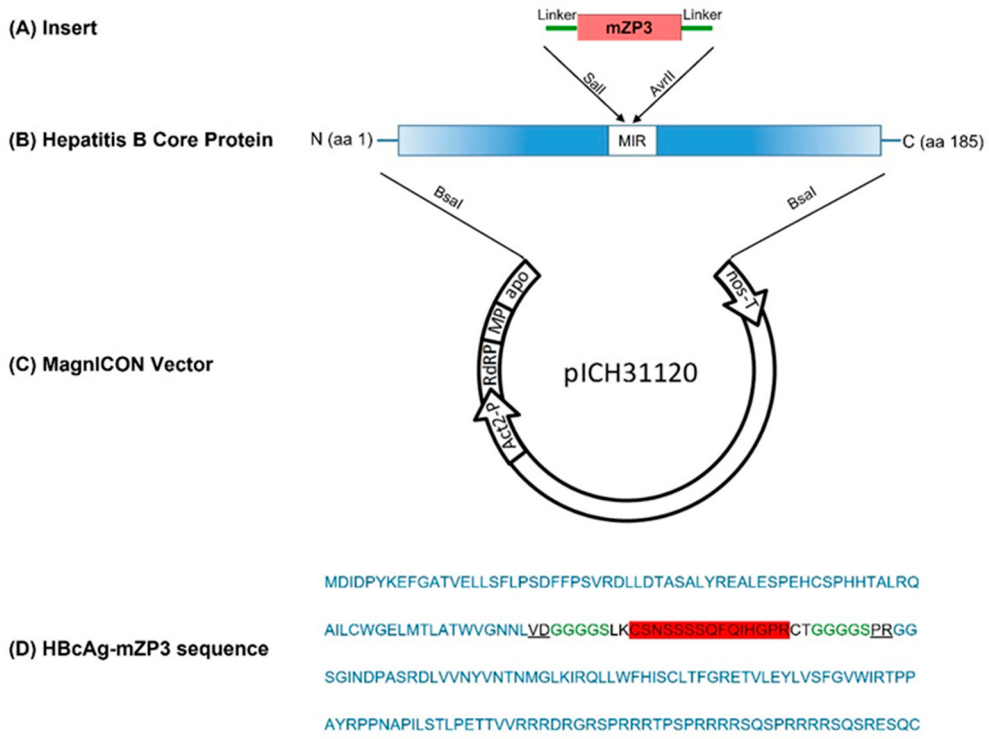

Figure 1. Schematic illustration of the recombinant vector, pICH-HBcAg-mZP3. Coding region for the mZP3-contraceptive epitope (**A**) inserted via flexible linkers (GGGGS) into the major immunodominant region (MIR) of full-length HBcAg (**B**). The MagnICON plant transient expression system was used in this study (**C**). Act2, Arabidopsis actin 2 promoter; RdRp, RNA-dependent RNA polymerase; MP, movement protein; apo: apple pectinase-apoplastidal targeting sequence; nos-T, nopaline synthase terminator. (**D**) Amino acid sequence of HBcAg-mZP3 construct. Amino acids of mZP3 peptide are shaded in red, flexible linkers are shown in green, and SalI and AvrII sites are underlined.

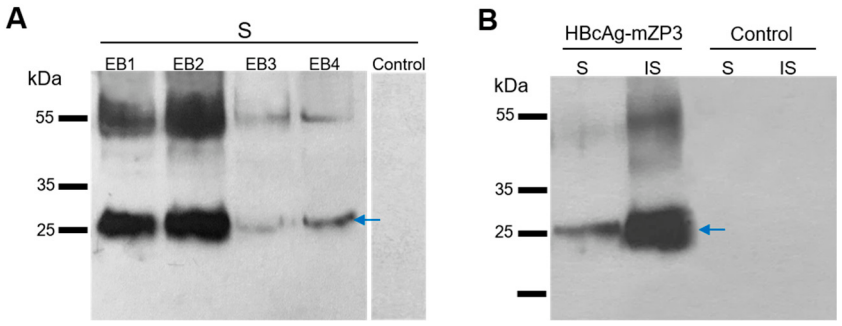

Figure 2. Western blot analysis of the HBcAg-mZP3 protein expressed in *N. benthamiana* leaves, identified in soluble (S) and insoluble (IS) fractions. (**A**) Effect of extraction buffer on soluble HBcAg-mZP3 extraction. Membrane probed with anti-mZP3 antibodies. (**B**) Detection and comparison of HBcAg-mZP3 protein in S and IS fractions extracted in EB2 condition. Extract from pICH31120-infiltrated leaves was used as control. Arrows indicate the monomeric form of HBcAg-mZP3. Blots cropped for clarity, for uncropped blots see Figure S2.

The infiltrated leaves were evaluated by Western blotting for soluble HBcAg-mZP3 accumulation at different time points. Western blotting of TSP extracted from leaves on days 4–10 post infiltration was probed with an anti-mZP3 antibody. Although a positive signal was detected from 4 dpi, the highest accumulation of HBcAg-mZP3 was observed between 8 and 9 dpi (Figure 3A). At each time point, the leaf phenotype was also documented. The symptoms of leaf necrosis were visible from 6 dpi and gradually worsened, leading to

severe wilting by 10 dpi (see Supplementary Figure S1 for phenotype of infiltrated leaf at 8 dpi). Furthermore, the expression and accumulation of recombinant HBcAg-mZP3 at different time points were quantified by ELISA using antibodies that recognized the mZP3 peptide. The expression of HBcAg-mZP3 reached the highest level at 8 dpi, with an average accumulation of 2633 µg/g leaf dry weight (LDW), corresponding to ~172 µg/g LDW mZP3 peptide, and then gradually decreased (Figure 3B). Based on the above observations, we set 8 dpi as the optimal harvest time for HBcAg-mZP3 expressed in *N. benthamiana*, using the MagnICON system.

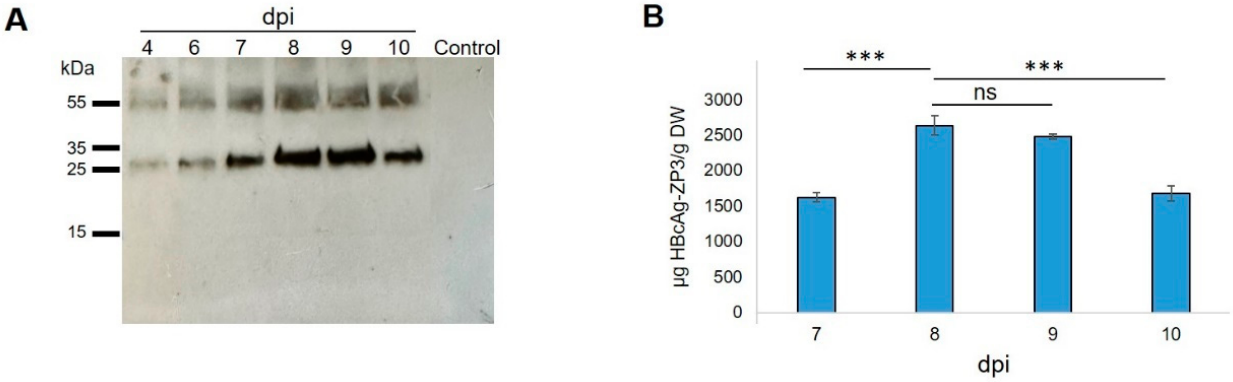

Figure 3. Western blot and ELISA analyses of HBcAg-mZP3 expression in agroinfiltrated *N. benthamiana* leaves from 4 dpi to 10 dpi. TSP was extracted from leaves on 4 dpi to 10 dpi. (**A**) Western blot analysis of protein extracts (20 µg TSP) probed with rabbit anti-mZP3 antibodies. Protein extracted from pICH31120-infiltrated leaves was used as control. dpi: days post infiltration. (**B**) Expression and accumulation of HBcAg-mZP3 were analyzed with ELISA of TSP extracted on 7–10 dpi. Error bars indicate standard deviation. *** indicates p values < 0.001. ns = non-significant difference ($p < 0.5$). See Figure S3 for uncropped blot. A Coomassie-stained gel of the leaf extract at 8 dpi is presented in Supplementary Material Figure S4.

3.3. Purification, Characterization, and Detection of Assembled HBcAg-mZP3 VLPs

Leaves infiltrated with HBcAg-mZP3 were harvested at 8 dpi to purify the HBcAg-mZP3. Sucrose density gradients were applied to determine whether the expression of HBcAg-mZP3 in the plant led to the formation of VLPs. In the first purification method, extracts from HBcAg-mZP3-infiltrated leaves were sedimented on 10–60% sucrose gradients (Figure 4A), fractionated, and monitored by Western blotting. Analysis of the fractions from the sucrose gradient revealed a protein band corresponding to the size of HBcAg-mZP3. As shown in Figure 4B, the majority of HBcAg-mZP3 was distributed in 30–40% sucrose fractions, similar to what is known for assembled VLPs.

In the second approach for sedimentation of HBcAg-mZP3, protein extracts containing HBcAg-mZP3 were loaded on a double sucrose gradient. After ultracentrifugation, a green band formed at the interface between the 25% and 70% sucrose layers (Figure 5A). Western blot analysis of the fractions showed that a major part of HBcAg-mZP3 sedimented towards the bottom of the tube (Supplementary Material Figure S5) and was mainly detected within the interface layer and the 70% sucrose layer below. The maximum HBcAg-mZP3 recovery was achieved when both the 70% fraction and the interface fraction were collected together (Figure 5B). A Coomassie-stained gel is presented in the Supplementary Material, Figure S4. These results strongly suggest that plant-produced HBcAg-mZP3 assembled into VLPs [64]. This method was used for preparing partially purified HBcAg-mZP3 for subsequent experiments.

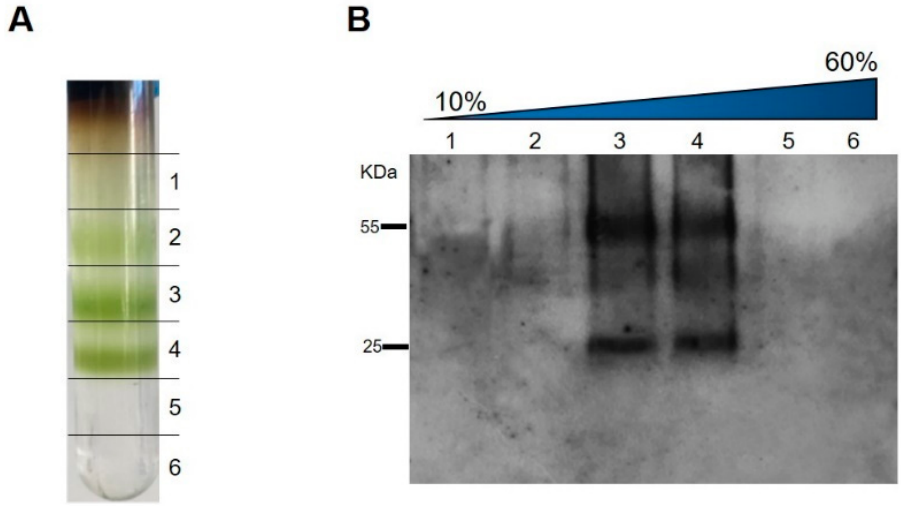

Figure 4. Gradient purification and detection of plant-produced HBcAg-mZP3. Total protein extract was subjected to a 10–60% sucrose gradient. (**A**) Photograph of an ultracentrifuge tube after ultracentrifugation. (**B**) Western blot analysis of sucrose gradient fractions. Sedimentation is left to right. An anti-mZP3 antibody was used to detect HBcAg-mZP3.

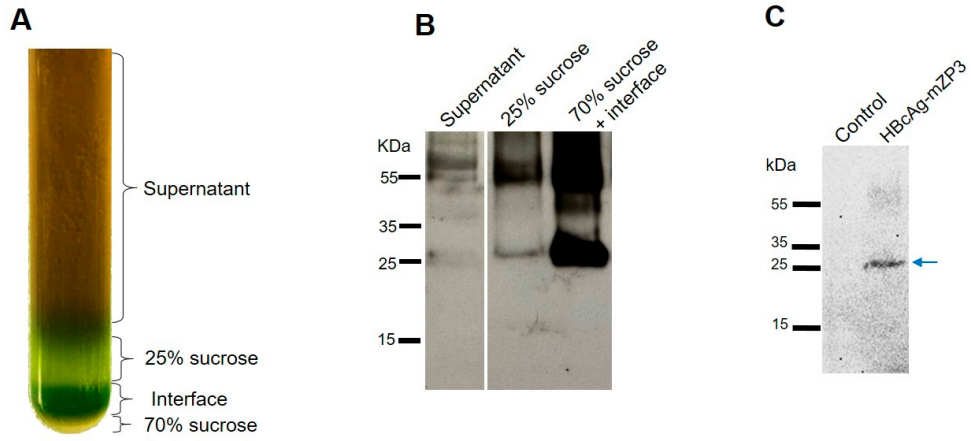

Figure 5. Partial purification of HBcAg-mZP3 VLPs using a double sucrose cushion. (**A**) Photograph of ultracentrifuge tube after ultracentrifugation. (**B**) HBcAg-mZP3 VLPs recovered from the interface and 70% fractions compared to other fractions. Western blots were probed with anti-mZP3 antibody to detect HBcAg-mZP3. (**C**) Lectin blot indicates binding of Con A with partially purified plant-produced HBcAg-mZP3. Lane 1, similarly prepared control sample (pICH31120-infiltrated leaves); lane 2, HBcAg-mZP3. Arrow indicates monomeric form of HBcAg-mZP3. Blots cropped for clarity, for uncropped blots see Figure S6.

Glycosylation of mZP3 was expected to be achieved by targeting HBcAg-mZP3 to the secretory pathway. Hence, a lectin blot assay was performed with peroxidase-conjugated concanavalin A (Con A), a carbohydrate-binding lectin, to investigate the possible glycosylation of plant-produced HBcAg-mZP3. The lectin blot of partially purified plant-produced HBcAg-mZP3 showed the reaction of HBcAg-mZP3 with Con A (Figure 5C), indicating the presence of sugar residues in the plant-produced HBcAg-mZP3, suggesting mZP3 glycosylation.

To confirm the formation of HBcAg-mZP3 VLPs, samples from the HBcAg-mZP3-rich fractions, 30–40% sucrose fractions collected from the sucrose density gradient and a 70% fraction from the double sucrose cushion, were subjected to negative staining and transmission electron microscopy (TEM). TEM observations confirmed that HBcAg-mZP3 produced in plants was successfully assembled into VLPs (Figure 6). The assembled HBcAg-mZP3 VLPs showed a typical shape and size similar to those documented in the literature for HBcAg VLPs.

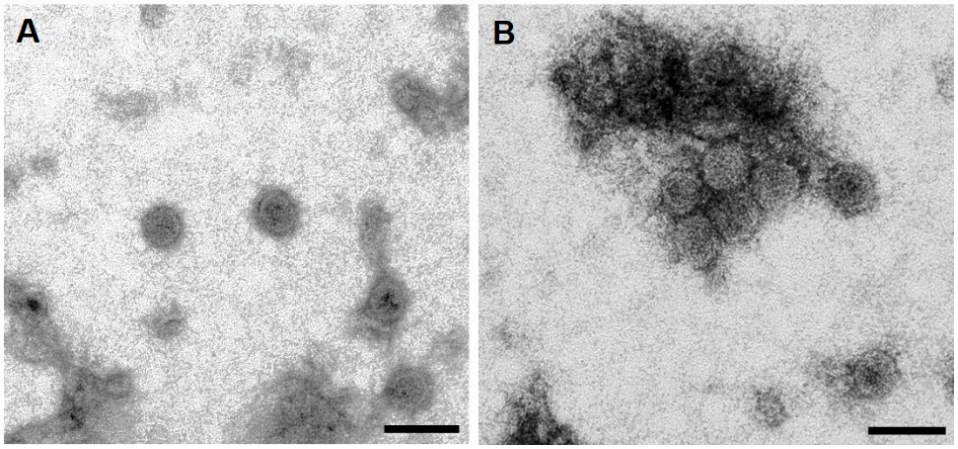

Figure 6. Electron microscopy of plant-produced HBcAg-mZP3 partially purified from leaf extract using sucrose gradients. TEM analysis of (**A**) HBcAg-mZP3 VLPs from 30–40% sucrose fractions, and (**B**) HBcAg-mZP3 VLPs from 70% sucrose fraction. Scale bars = 50 nm. A larger field image is presented in Supplementary Material Figure S7.

3.4. Systemic Immunogenicity of Plant-Produced HBcAg-mZP3 in Mice

Because of the difficulties of maintaining wild-type mice in the laboratory, we examined the immunogenicity of mZP3 displayed on HBcAg VLPs in BALB/c mice. Subcutaneous immunization of mice was performed using partially purified plant-produced HBcAg-mZP3. The first group (n = 5) was immunized subcutaneously with three doses of HBcAg-mZP3 VLPs containing 5.5 µg mZP3 and 10% Polygen as an adjuvant, at three-week intervals. The control group was injected with saline buffer (PBS) with 10% Polygen. Serum samples were collected before immunization and on days 21, 36, and 57 (Figure 7A), and anti-mZP3 antibodies were measured in the sera from individual mice. In an indirect ELISA using a purified plant-produced mZP3 as the coating antigen, anti-mZP3 IgG antibodies were not detected in pre-immune serum or in sera after the prime dose (day 21). However, HBcAg-mZP3 elicited IgG antibody responses to mZP3 in immunized mice after the first booster dose, and these IgG antibody responses to mZP3 were significantly higher than those in the control group ($p < 0.0001$). A significant further boosting effect was observed after the last immunization dose, which caused significant ($p < 0.01$) elevations in the anti-mZP3 IgG responses, although there was variation between individual immunized mice (Figure 7B). While no anti-mZP3 response was observed throughout the immunization course in the control group, anti-mZP3 IgG was detectable in the sera of HBcAg-mZP3-immunized mice at serum dilutions over $1{:}10^5$ after booster doses. Pooled mice sera (n = 5) collected after the final subcutaneous immunization showed a mean IgG antibody titer of 1,000,000 (Supplementary Material, Figure S8A).

3.5. Mucosal Immunogenicity of Plant-Produced HBcAg-mZP3 in Mice

For the oral immunization of mice, 10 animals received a suspension of 120 mg lyophilized leaves expressing HBcAg-mZP3 (containing approximately 190 µg mZP3). Mice in the control group were administered similarly prepared control samples (pICH31120-infiltrated leaves). Sera and fecal samples were collected before immunization and after each immunization (Figure 8A) to measure the anti-mZP3 IgG and IgA antibodies. IgG anti-mZP3 antibodies were detected in sera after the second oral immunization, and the response increased significantly ($p < 0.05$) with the subsequent dose (Figure 8B). Furthermore, a higher boosting effect was observed after the last oral immunization, which significantly ($p < 0.0001$) increased specific anti-mZP3 IgG responses. The response detected in the control group was similar to that in the serum samples collected before the first immunization (Figure 8B). After the final oral immunization, the mean endpoint titers of anti-mZP3 IgG in the pooled sera (n = 10) reached the value of 10,000 (Supplementary Material, Figure S8B).

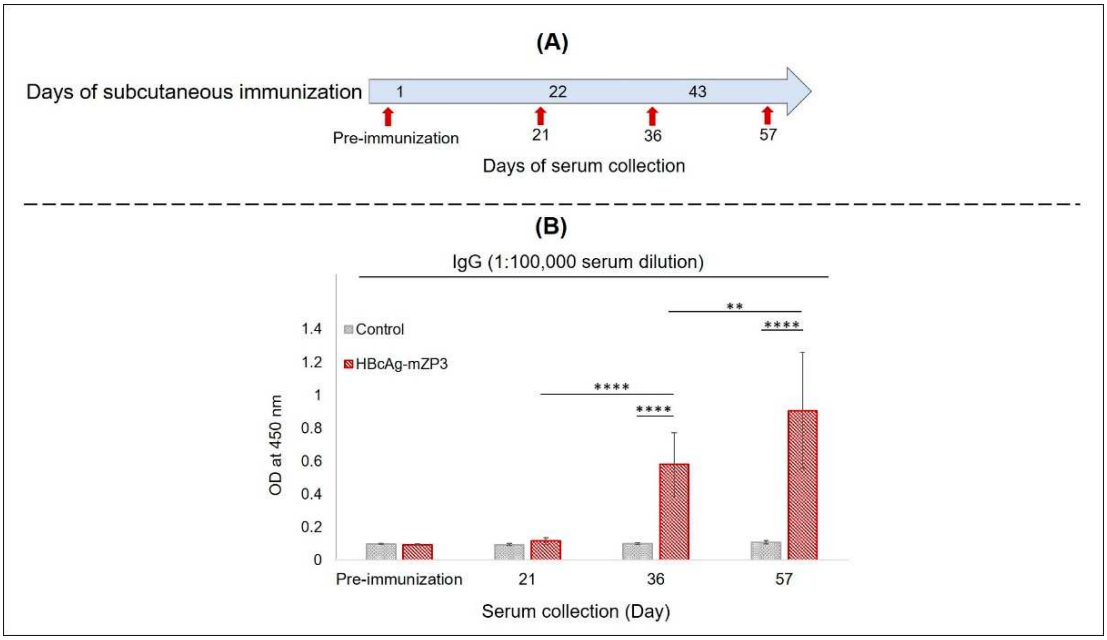

Figure 7. Evaluation of mean anti-mZP3 antibody responses in immunized BALB/c mice using ELISA. BALB/c mice subcutaneously immunized with plant-produced HBcAg-mZP3. (**A**) Immunization and sampling schedule. (**B**) Anti-mZP3 IgG antibody levels measured in sera before immunization and after each immunization. Data presented as mean ± SD of absorbance values at 450 nm. ** $p < 0.01$ and **** $p < 0.0001$.

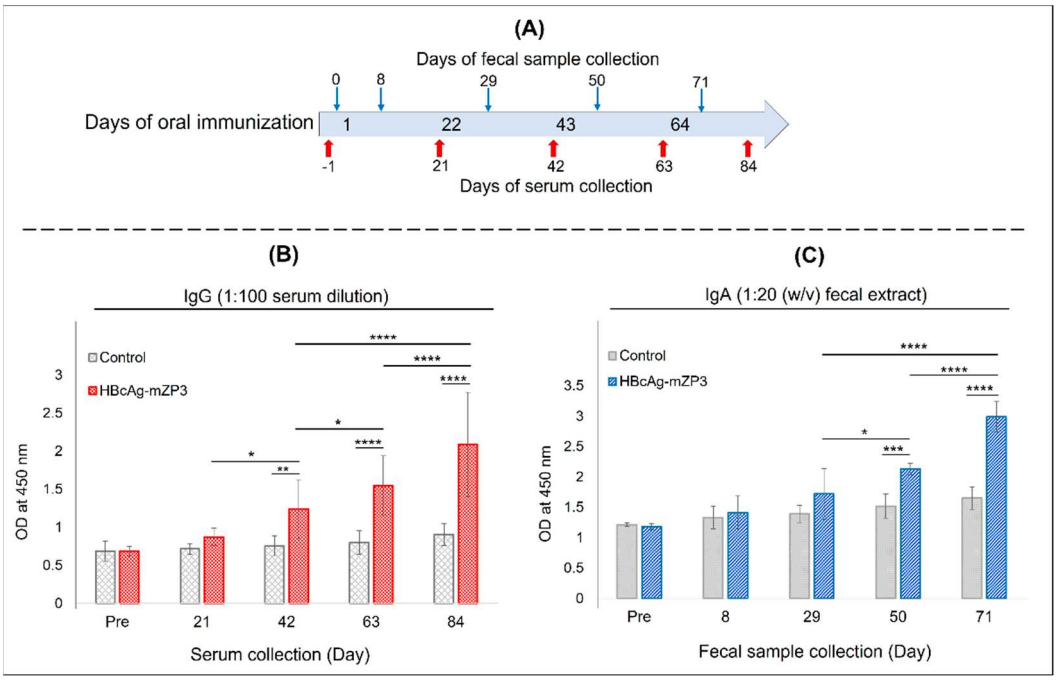

Figure 8. Evaluation of mean anti-mZP3 antibody responses in BALB/c mice orally immunized with plant leaves producing HBcAg-mZP3. (**A**) Immunization and sampling schedule. (**B**) Anti-mZP3 IgG antibody levels measured in sera before immunization and after each immunization. (**C**) Anti-mZP3 IgA antibodies in feces extract measured in samples collected before and seven days after each oral immunization. Data presented as mean ± SD of absorbance values at 450 nm. * indicates $p < 0.05$; ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$.

No significant IgA responses to mZP3 were detected in the fecal samples after the first two oral administrations of HBcAg-mZP3 compared to the pre-immunization and control groups (Figure 8C). However, the IgA response in fecal samples increased after the third oral dose of HBcAg-mZP3 and displayed a significant difference from that after the second dose ($p < 0.05$) and the control group ($p < 0.001$). As shown in Figure 8C, the fourth dose of the HBcAg-mZP3 oral vaccine markedly increased the mucosal IgA response in mice, which was significantly ($p < 0.0001$) higher than that of the third dose (8C). The responses detected in the control group may be due to the presence of antibodies against contaminating plant proteins [66]. The mean endpoint titer of anti-mZP3 IgA in pooled fecal extracts was $\geq$ 40 (w/v) and significant versus the control group (Supplementary Material, Figure S8C).

3.6. Binding of Antibodies to Native Zona Pellucida In Vitro

Serum IgG and mucosal IgA antibodies from HBcAg-mZP3-treated mice were used in an indirect immunofluorescence assay on wild mouse ovarian sections to evaluate the binding of antibodies to the ZP matrix. These observations showed that anti-mZP3 IgG antibodies generated in subcutaneously immunized female mice cross-reacted with the native ZP matrix in wild mouse ovaries (Figure 9A). Immune sera of orally immunized mice also reacted with the ZP of wild mice (Figure 9B). Furthermore, mucosal IgA antibodies from orally immunized mice specifically recognized and bound to the ZP of wild-type mice, and fluorescence was strongly observed around the oocytes (Figure 9C).

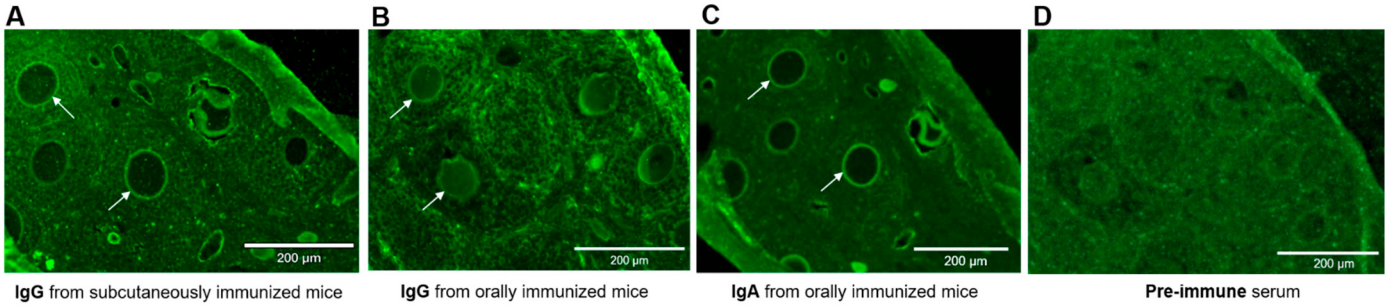

Figure 9. Indirect immunofluorescence microscopy to detect the binding of antibodies against HBcAg-mZP3 to ZP matrix. Sections from the same ovary were used. Wild mouse ovarian sections treated with (**A**) serum from subcutaneously immunized mice, (**B**) serum from orally immunized mice, and (**C**) fecal extracts from orally immunized mice. (**D**) Control experiments were performed with pre-immune serum. Arrows point to antibodies that reacted with the zona pellucida. Scale bars = 200 µm.

4. Discussion

The reduction of mouse fertility in the field requires an orally applicable, mouse-specific agent. The results described here provide a first step in this direction. They indicate that plants are a feasible platform for the production of sufficient quantities of putative mouse-specific mZP3 peptides. Plant-made mZP3 elicited ZP-specific antibody responses in immunized mice not only after subcutaneous administration, but also after oral administration. This study presented the oral immunogenicity of the mZP3 peptide for the first time. The induced antibodies cross-reacted with and bound to the wild mouse zona pellucida. Therefore, the efficacy of plant-produced mZP3 as an oral contraception vaccine to reduce the fertility of wild mice should be investigated.

Immunocontraception has been accepted as an alternative approach for managing overpopulated rodents and to address most concerns about the impact of rodenticides or other lethal methods of control on ecology and non-target species [3,15]. The mZP3 peptide produced in this study is not immunologically present in the ovaries of species such as guinea pigs, hamsters, cats, and dogs [33,67] and has been considered as a target for developing a mouse-specific contraceptive vaccine [26,28]. However, the species specificity of the vaccine to be administered orally should be verified. Ovarian damage or autoimmune

oophoritis, which can be a concern of immunization with zona pellucida antigens [68], was not observed in this study in the presence of mZP3 peptide-induced autoantibodies.

The successful production of the small peptide mZP3 in the form of HBcAg-mZP3 in plants helps to address issues associated with the cost and scalability of vaccine production. We previously described the production of an immunogenic single mZP3 peptide in *N. benthamiana* plants [65]. As expected, the production of HBcAg-mZP3 protein resulted in a significant increase in mZP3 expression compared to the unconjugated mZP3 peptide. This is consistent with our earlier observations, which showed that mZP3 expression was significantly improved by fusion with a stable partner such as GFP [65]. HBcAg-mZP3 expression resulted in high production of the mZP3 peptide, similar to that produced by the polypeptide antigen mZP3–3, which contains three repeats of the mZP3 peptide [65]. The addition of DTT and Triton X-100 to the extraction buffer [63,69,70] increased the yield of soluble HBcAg-mZP3, and up to 2.63 mg/g dry weight HBcAg-mZP3 was detected. However, regardless of the buffer used, the HBcAg-mZP3 protein was mainly present in the insoluble fraction, which has also been reported for recombinant cytosolic HBcAg [71,72]. The formation of HBcAg-mZP3 virus-like particles was expected based on the distribution of HBcAg-mZP3 in particular sucrose gradient fractions [63,64,73] and was confirmed by electron microscopy. Despite the insertion of mZP3 into the MIR of HBcAg and targeting the protein to the secretory pathway, HBcAg-mZP3 assembled into VLPs similar to what has been observed for cytosolic wild-type HBcAg VLPs [63,73] and cytosolic [74] and ER-targeted [75,76] chimeric HBcAg VLPs. Hence, it can ensure the repetitive presentation of glycosylated mZP3 on the surface of assembled VLPs to elicit strong immune responses.

HBcAg was chosen to display mZP3 because several studies have reported the enhanced immunogenicity of small peptides conjugated to HBcAg VLPs [44,74]. Our results revealed that subcutaneous administration of plant-produced HBcAg-mZP3 elicited high humoral antibody responses against the mZP3 peptide compared to synthetic mZP3 [31] or *E. coli*-produced mZP3 peptides [26]. These results suggest that mZP3 can be readily displayed on the surface of particles and recognized by the immune system. Although the dose of mZP3 delivered in the form of HBcAg-mZP3 was approximately 10% of the HBcAg-free antigens used in our earlier study [65], the level of induced antibodies was higher than that of the single-peptide antigen, GFP-mZP3, and comparable to that of the polypeptide antigen, mZP3–3 (Supplementary Material, Figure S9). These results underscore the potential adjuvant effect of HBcAg VLP in enhancing the strong immune responses against mZP3. A reduction in the necessary dose to induce a strong immune response was expected because HBcAg VLPs have immune-enhancing properties and convey high immunogenicity to the heterologous epitope [41,44]; therefore, a lower dose of chimeric antigen (epitope) is usually sufficient [37,77]. Moreover, the high density of the mZP3 epitope repetitively displayed at the MIR, which is the most exposed and immunogenic region of particles, enhances immune responses against this heterologous epitope [39,44,78].

Transgenic plants can provide suitable formulations for the oral delivery of vaccines and reduce the costs of vaccine production significantly [51,52,79]. The results of this study indicate that repeated oral administration of plant cells containing HBcAg-mZP3 VLPs not only could evoke humoral immunity, but also was able to induce mucosal antibody responses against mZP3 in immunized mice. It has already been demonstrated that oral immunization of mice with a recombinant ZP3 protein could induce significant levels of IgG and IgA antibodies, leading to reduced fertility [80], and plant-produced HBcAg VLPs have also been shown to be capable of stimulating IgG and IgA responses in mucosal delivery [73,81]. This study is the first report of oral immunogenicity of the mZP3 peptide. Oral immunization with the small mZP3 in the form of HBcAg-mZP3 led to the induction of IgG and IgA antibodies that recognized the native ZP in wild mice. Hardy et al. [31] have shown that inducing antibodies cross-reacting with mZP3 resulted in reduced fertility in wild mice. Therefore, binding of induced antibodies to the native ZP of wild mice in vitro might reflect binding to the wild mouse ZP in vivo. Hence, further research is

recommended to investigate the contraceptive efficacy of plants producing HBcAg-mZP3 VLPs as oral baits in wild mice.

Oral administration of the fourth dose of the plant tissue containing HBcAg-mZP3, without any additional adjuvants, induced a significant response to mZP3 compared with the third dose, which clearly indicated a boosting effect. It has been reported that oral contraceptive vaccines could be effective for a long time after multiple immunizations by inducing mucosal immunity in addition to systemic immunity [80,82]. Some reports have shown that the oral delivery of repeated or higher doses of antigens induces a significant and long-term immune response [83,84]. On the other hand, others supported less frequent and lower doses of oral antigens for inducing an active immune response [85,86] because higher doses or continuous oral administration of antigens can result in significantly stronger oral tolerance [87]. Therefore, the dose and frequency of HBcAg-mZP3 administration must be determined to establish an effective regime for inducing efficient immune responses.

In summary, we demonstrated the successful production of glycosylated mZP3 presented on HBcAg VLPs. We showed that subcutaneous and oral delivery of plant-produced HBcAg-mZP3 can stimulate anti-mZP3 antibodies in BALB/c mice. Both anti-mZP3 IgG and IgA antibodies showed reactivity and binding to the zona pellucida in the ovaries of wild mice. It is therefore likely that plant-produced mZP3 is capable of inducing zona pellucida-reactive antibodies, which may inhibit fertility in wild-type mice. Nevertheless, this has to be proven by the oral delivery of feed pellets containing plant-expressed HBcAg-mZP3 protein to wild mice. In addition, species specificity must be verified to ensure that the vaccine can be safely released into the environment without affecting non-target species.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/vaccines11020462/s1, Figure S1: Phenotype of *N. benthamiana* leaves infiltrated with HBcAg-mZP3 at 8 dpi; Figure S2: Western blot analysis of the HBcAg-mZP3 protein expressed in *N. benthamiana* leaves, identified in soluble (S) and insoluble (IS) fractions. Figure S3. Western blot and ELISA analyses of HBcAg-mZP3 expression in agroinfiltrated *N. benthamiana* leaves from 4 dpi to 10 dpi. Figure S4: Coomassie-stained SDS-PAGE of extracts of *N. benthamiana* leaves. Figure S5: Partial purification of HBcAg-mZP3 VLPs using a double sucrose cushion. Figure S6: Partial purification of HBcAg-mZP3 VLPs using a double sucrose cushion and Lectin blot assay. Figure S7: Electron micrograph of HBcAg-mZP3 VLPs produced in *N. benthamiana*. Figure S8: Mouse mZP3-specific antibody titers. Figure S9: Comparison of serum antibody responses in mice immunized with mZP3 in the form of HBcAg-mZP3, GFP-mZP3–1, and mZP3–3 after the final boost injection.Figure S10: The endpoint titer ELISA data. The absorbance (OD) of each sample was read three times at 450 nm.

Author Contributions: K.G. designed and performed the experiments, analyzed the data, and wrote the manuscript. I.B. and J.H. supervised the study, provided scientific advice, and revised the manuscript. J.S. supervised the histological and immunofluorescence analyses. R.K. and N.K. performed animal experiments. A.S. performed electron microscopy. All authors have read and agreed to the published version of the manuscript.

Funding: The plant work was funded by a scholarship from the "Landesgraduiertenförderung Mecklenburg-Vorpommern" of the University of Rostock.

Data Availability Statement: The datasets generated during this study are available from the corresponding author upon request.

Acknowledgments: We are grateful for the support of Udo Meyer and Susanne Montag (Bioserv GmbH, Rostock, Germany) for the production of anti-mZP3 antibodies and their assistance during the animal trial. Additionally, we thank Patrick Inomoto and Mahshid Monavari (Innocent Meat GmbH, Rostock, Germany) for kindly providing the fluorescence microscope and their assistance. We would also like to thank Kerstin Thoss for technical assistance with ELISAs and Sonja Konopka for delivering the plants.

Conflicts of Interest: The authors declare no conflict of interest.

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Supplementary Material to 2.2

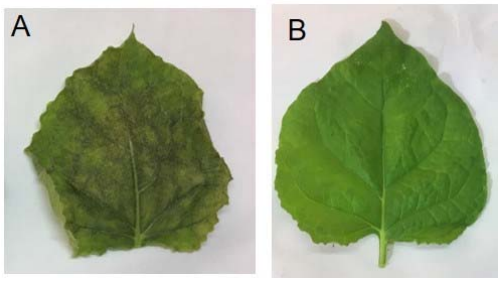

Figure 2.2 S1: Phenotype of *N. benthamiana* leaves infiltrated with HBcAg-mZP3 at 8 dpi (**A**) compared to un-infiltrated leaf (**B**).

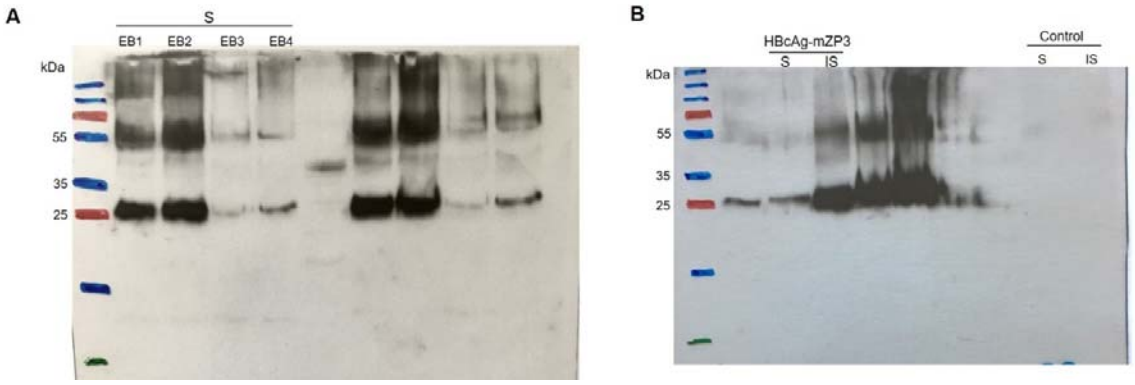

Figure 2.2 S2: Western blot analysis of the HBcAg-mZP3 protein expressed in *N. benthamiana* leaves, identified in soluble (S) and insoluble (IS) fractions. Figure 2 was cropped from these western blot images.

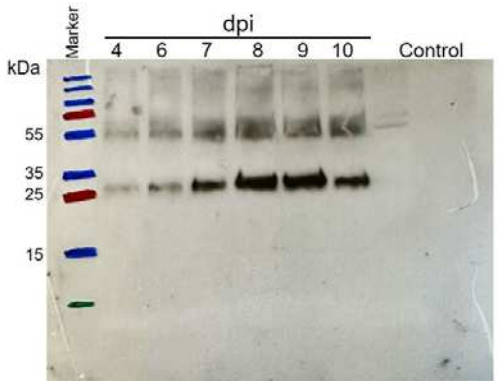

Figure 2.2 S3: Western blot and ELISA analyses of HBcAg-mZP3 expression in agroinfiltrated *N. benthamiana* leaves from 4 dpi to 10 dpi. Figure 3 was cropped from this western blot image.

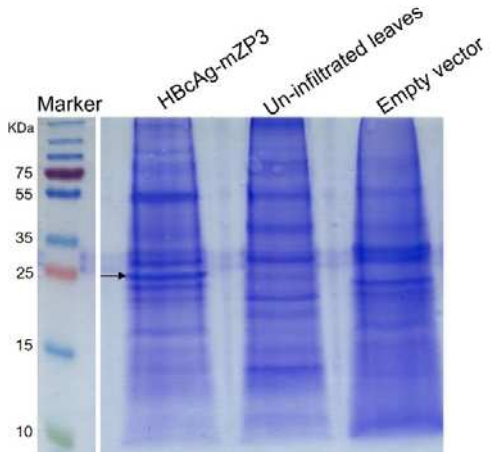

Figure 2.2 S4: Coomassie stained SDS-PAGE of extracts of *N. benthamiana* leaves infiltrated with HBcAg-mZP3 (8 dpi), un-infiltrated leaves, and leaves infiltrated with pICH31120 empty vector. The protein band around 25 kDa could be HBcAg.

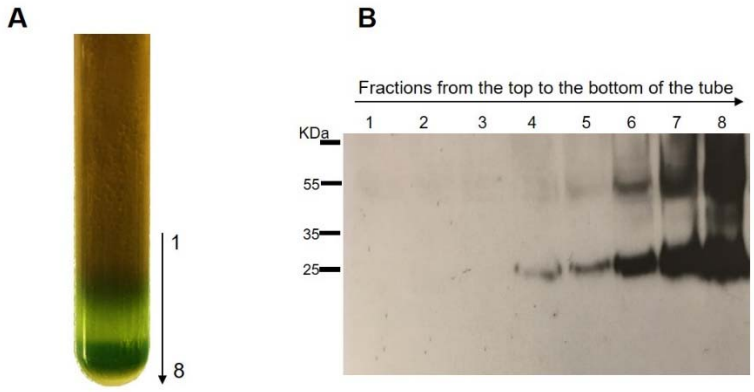

Figure 2.2 S5: Partial purification of HBcAg-mZP3 VLPs using a double sucrose cushion. (A) Photograph of ultracentrifuge tube after ultracentrifugation. (B) Eight fractions (8 x 0.5 ml) were collected from the bottom of the tubes and analyzed by western blot.

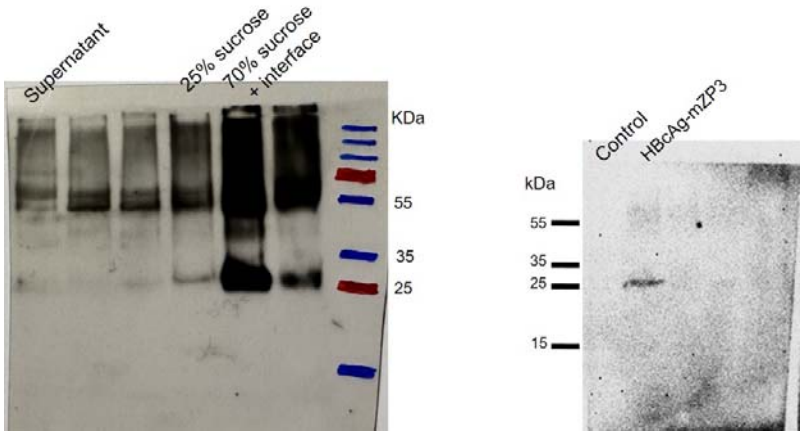

Figure 2.2 S6: Partial purification of HBcAg-mZP3 VLPs using a double sucrose cushion and Lectin blot assay. Figure 5 was cropped from these western blot images.

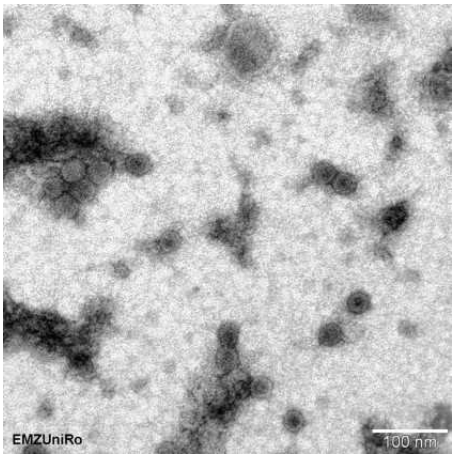

Figure 2.2 S7: Electron micrograph of HBcAg-mZP3 VLPs produced in *N. benthamiana*. Bar = 100 nm.

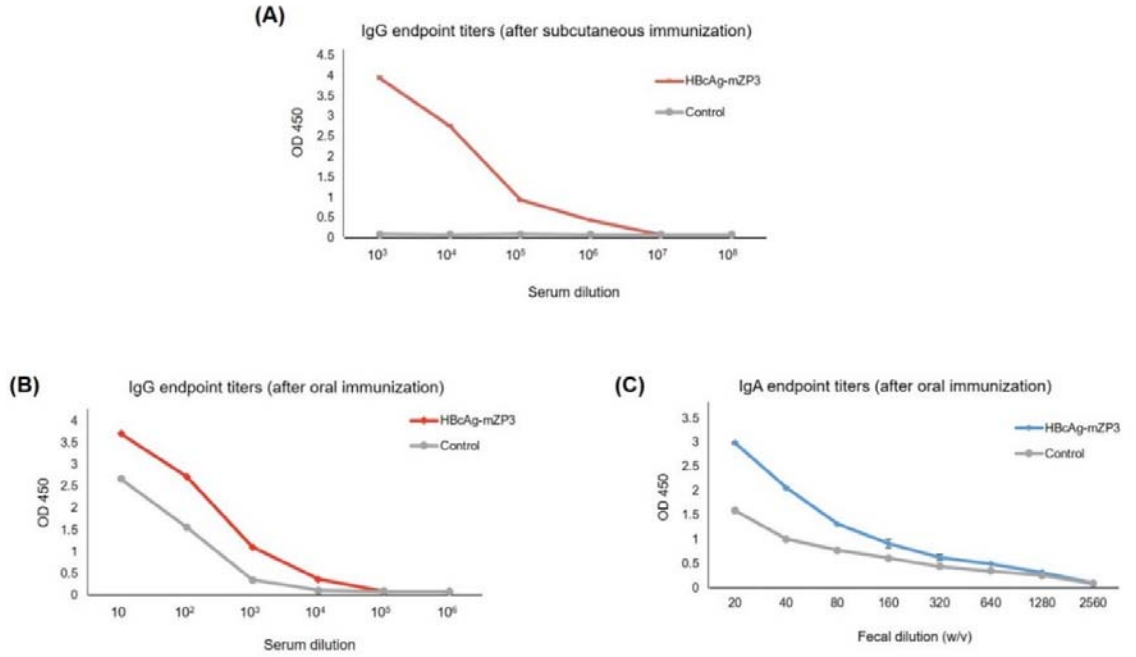

Figure 2.2 S8: Mouse mZP3-specific antibody titers. (A) Pooled sera collected after the final subcutaneous immunization (n = 5), (B) pooled sera (n = 10), and (C) pooled fecal extracts (n = 10) collected after the final oral immunization, were analyzed for anti-mZP3 antibodies by endpoint titer ELISA. Error bars indicate the standard deviation of triplicate ELISA readings. The standard deviation of most data is small and invisible (See figure S10 for ELISA reading data).

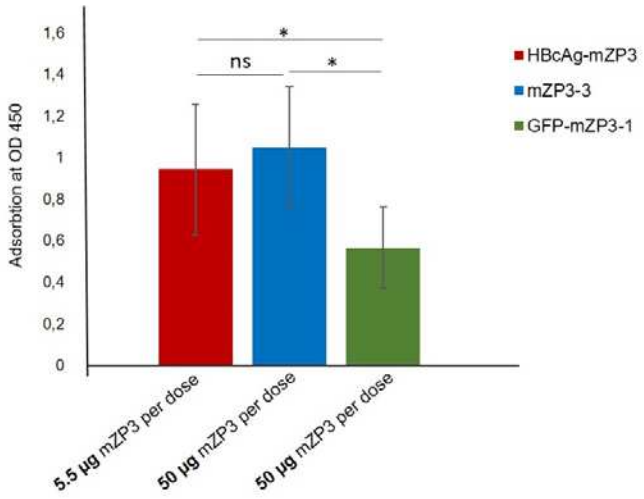

Figure 2.2 S9: Comparison of serum antibody responses in mice immunized with mZP3 in the form of HBcAg-mZP3, GFP-mZP3-1 and mZP3-3 after the final boost injection. Absorbance values are shown for sera of each immunized group at a 1:100,000 dilution. Data are presented as the mean ± SD absorbance values at 450 nm. *$p < 0.05$; ns = non-significant difference ($p < 0.5$).

IgG, Subcutaneous immunization							
		HBcAg-mZP3			Control		
		OD1	OD2	OD3	OD1	OD2	OD3
Serum dilution	10^3	3.914	3.948	3.977	0.086	0.084	0.084
	10^4	2.755	2.766	2.761	0.082	0.083	0.084
	10^5	0.924	0.940	0.932	0.085	0.087	0.083
	10^6	0.429	0.440	0.435	0.081	0.078	0.082
	10^7	0.084	0.082	0.083	0.079	0.078	0.080
	10^8	0.071	0.078	0.075	0.074	0.081	0.076

IgG, Oral immunization							
		HBcAg-mZP3			Control		
		OD1	OD2	OD3	OD1	OD2	OD3
Serum dilution	10	3.709	3.698	3.704	2.702	2.620	2.667
	10^2	2.735	2.706	2.721	1.590	1.526	1.552
	10^3	1.123	1.090	1.107	0.338	0.373	0.356
	10^4	0.395	0.338	0.367	0.114	0.114	0.114
	10^5	0.096	0.094	0.095	0.096	0.087	0.092
	10^6	0.093	0.089	0.091	0.107	0.095	0.100

IgA, Oral immunization							
		HBcAg-mZP3			Control		
		OD1	OD2	OD3	OD1	OD2	OD3
Fecal dilution	20	2.995	2.995	2.964	1.617	1.606	1.621
	40	2.040	2.090	2.065	1.005	1.022	1.011
	80	1.316	1.328		0.784	0.776	0.780
	160	0.829	1.004	0.917	0.625	0.606	0.616
	320	0.691	0.576	0.634	0.423	0.472	0.448
	640	0.499	0.501	0.500	0.347	0.363	0.355
	1280	0.325	0.327	0.321	0.268	0.262	0.265
	2560	0.108	0.115	0.112	0.088	0.107	0.098

Figure 2.2 S10: The endpoint titer ELISA data. The absorbance (OD) of each sample was read three times at 450 nm.

2.3 Immunogenicity and contraceptive efficacy of plant-produced putative mouse-specific contraceptive peptides

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Published in

Front. Plant Sci. (2023), 14:1191640.

Sec. Plant Biotechnology

DOI: https://doi.org/10.3389/fpls.2023.1191640

frontiers | Frontiers in Plant Science

TYPE Original Research
PUBLISHED 28 June 2023
DOI 10.3389/fpls.2023.1191640

OPEN ACCESS

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RECEIVED 22 March 2023
ACCEPTED 09 June 2023
PUBLISHED 28 June 2023

CITATION
Ghasemian K, Broer I, Schön J, Kolp N, Killisch R, Mikkat S and Huckauf J (2023) Immunogenicity and contraceptive efficacy of plant-produced putative mouse-specific contraceptive peptides. *Front. Plant Sci.* 14:1191640. doi: 10.3389/fpls.2023.1191640

Immunogenicity and contraceptive efficacy of plant-produced putative mouse-specific contraceptive peptides

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Rodent population control through contraception requires species-specific oral contraceptive vaccines. Therefore, in this study, we produced putative mouse-specific contraceptive peptides, mZP2 (from oocyte) and mIzumo1 (from sperm), in plants using *Agrobacterium*-mediated transient expression. Peptides were produced separately in *Nicotiana benthamiana* using constructs encoding antigens containing three copies of each peptide. We also determined the immunogenicity and contraceptive effects of the plant-produced antigens in female BALB/c mice. Mice immunized subcutaneously with a relatively low amount of antigen (5 µg/dose of each peptide in a mixture) showed systemic immune responses against mZP2-3 and mIzumo1-3 antigens. Moreover, the mean litter size of mice treated with the plant-produced antigens was reduced by 39% compared to that of the control mice. Notably, there was a significant negative correlation between the number of pups born and individual antibody levels against both antigens. Immunofluorescence assays demonstrated the binding of induced antibodies to the oocytes of BALB/c and wild-type mice *in vivo* and *in vitro*, respectively. Our study demonstrate the feasibility of producing small contraceptive peptides in plants that can be further used to develop oral contraceptive vaccines against mouse populations.

KEYWORDS

plant molecular farming, *Nicotiana benthamiana*, contraceptive vaccine, Zona pellucida, Izumo

1 Introduction

Increased population of rodent pests, such as mice, causes economic and environmental damage worldwide (Singleton et al., 2010; Wondifraw et al., 2021; Brown and Henry, 2022). Therefore, effective population management strategies are needed to control these pests (Jacob, 2008; Jacoblinnert et al., 2021). Fertility control is considered a safer alternative to currently used methods, such as culling, trapping, and poisoning, which are unethical, stimulate reproduction, and pose a risk to non-target species (Barfield et al., 2006; Singleton et al., 2007; Imakando et al., 2021). Immunocontraception is the use of vaccines to reduce fertility by inducing immune responses against essential proteins involved in various stages of fertilization Chambers et al., 1997; Barber and Fayrer-Hosken, 2000; Naz, 2005). Several egg- and sperm-specific candidate antigens capable of inhibiting fertility have been identified [reviewed in (Naz, 2005; Gupta and Minhas, 2017)].

Proteins of Zona pellucida (ZP), an extracellular glycoprotein matrix covering the mammalian oocytes, are most commonly used as contraceptive vaccine antigens (Barber and Fayrer-Hosken, 2000; Gupta et al., 2004). ZP2, the secondary receptor of sperm, is an essential protein for reproduction (Bleil et al., 1988; Wassarman, 1988), and antibodies against its epitopes interfere with sperm-binding and inhibit fertilization in mice (Hinsch et al., 1998; Sun, 1999; Hardy et al., 2008). In addition to oocyte-specific antigens, several spermatozoa-specific antigens have been proposed as targets for immunocontraception. Izumo1 is a sperm-specific protein localized in the acrosomal region of sperm that plays a vital role in sperm–egg fusion and fertilization (Inoue et al., 2005; Naz, 2014; Xue et al., 2016). A previous study demonstrated that sperms produced by Izumo1-deficient mice are unable to fuse with eggs (Inoue et al., 2005). Izumo1 is exposed and accessible in female reproductive tract after the acrosome reaction (Inoue et al., 2005). Izumo1 can induce immune responses in the serum and genital tracts of immunized mice (Naz, 2008). Immunization with vaccines based on mouse Izumo1 significantly decreases the fertility and litter size of mice (Naz, 2008; An et al., 2009; Wang et al., 2009).

Peptide-based contraceptive immunogens can improve the safety and species-specificity of vaccines (Hinsch et al., 1999; Ferro and Mordini, 2004; Hardy et al., 2004), which are crucial for vaccines intended to target wildlife populations (Bradley et al., 1999; Kirkpatrick et al., 2011). ZP proteins are not species-specific (Frank et al., 2005; Naz, 2005); therefore, antigens must be reduced to the species-specific regions of the oocyte and sperm proteins. Targeting peptides from functional and less conserved regions of contraceptive proteins may be a promising approach to achieve species-specificity (Lou et al., 1995; Hinsch et al., 1999; Hardy et al., 2008). Contraceptive and putative mouse-specific peptides, mZP2 (Sun, 1999; Hardy et al., 2008) and mIzumo1 (Naz, 2008; Xue et al., 2016), are effective in reducing the fertility of female mice. Peptide vaccines are less effective compared with protein vaccines (Ferro and Mordini, 2004). However, incorporation of adjuvants and multiple epitopes into antigens can improve the immunogenicity and efficacy of peptide-based antigens (Sadler et al., 2002; Ferro and Mordini, 2004; Hardy et al., 2004; Ghasemian et al., 2023a). Production of peptide-based mouse-specific contraceptive antigens facilitates the oral administration of vaccines to widely dispersed animals.

Transgenic plants expressing immunocontraceptive vaccines provide a safe production and delivery system. In addition to their simple production and easy scale-up processes, plant expression systems present a minimal risk of toxicity and contamination with mammalian pathogens (Daniell et al., 2009; Kwon et al., 2013). Use of plant-based contraceptive vaccines provides a relatively cost-effective control strategy to target free-ranging wildlife species (Smith et al., 1997; Polkinghorne et al., 2005). These vaccines can be further formulated as oral baits or used directly as feed (Daniell et al., 2009), enabling the widespread application of contraceptives for mouse population management.

In this study, we developed a proof-of-principle for the use of plants as systems for the production of contraceptive peptides. We used a transient expression system (viral-based magnICON system) to produce mZP2 and mIzumo1 peptides, from mouse ZP2 and Izumo1 proteins, respectively, in *Nicotiana benthamiana*. We also assessed the immunogenicity of plant-produced antigens in immunized mice as well as the fertility of vaccinated mice. Moreover, immunohistochemistry was used to investigate the *in vivo* and *in vitro* reactivities of the induced antibodies.

2 Materials and methods

2.1 Construction of plant expression vectors

Coding sequences of mZP2-3 (containing three copies of mZP2 peptide, TDVRYKDDMYHFFCPAIQA) and mIzumo1-3 (containing three copies of mIzumo1 peptide, LDCGERHIEVHRSEDLVLDCL) constructs (Figure 1) were designed with optimized codons for expression in *N. benthamiana*. Gene constructs were synthesized by Eurofins Genomics GmbH (Ebersberg, Germany) and cloned into the pEX-A vector. Coding regions were flanked by BsaI restriction sites after polymerase chain reaction (PCR) amplification. PCR products were cloned separately into the pJET1.2 cloning vector (CloneJET PCR Cloning Kit; Thermo Fisher Scientific, Waltham, Massachusetts, USA). Coding sequences were cloned into a tobacco mosaic virus (TMV)-based expression vector of the MagnICON transient expression system, pICH31120, with BsaI restriction and ligation (Engler et al., 2008). MagnICON vectors were kindly provided by Nomad Bioscience GmbH (Halle/Saale, Germany).

2.2 Transient expression of mZP2-3 and mIzumo1-3 in *N. benthamiana* leaves

Recombinant expression vectors were separately introduced into *Agrobacterium tumefaciens* strain ICF320 via electroporation. The resulting transformed colonies (confirmed using colony PCR

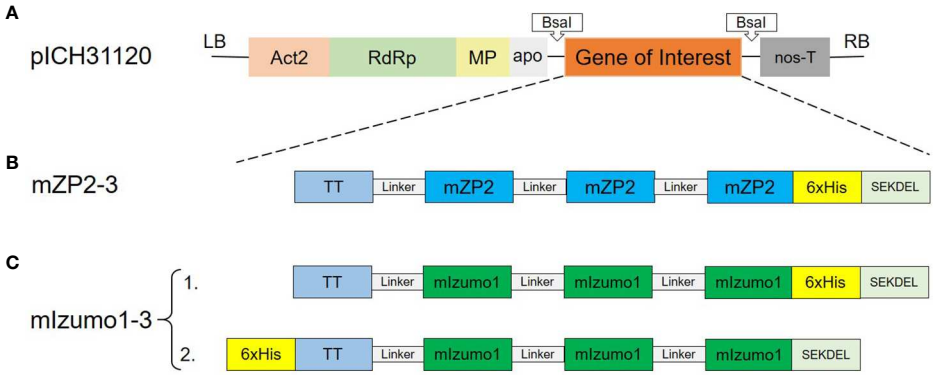

FIGURE 1
Schematic representation of the T-DNA region of the MagnICON vector and expression constructs used in this study. **(A)** Binary pICH31120 expression vector. Act2, *Arabidopsis* actin 2 promoter; RdRp, RNA-dependent RNA polymerase; MP, tobacco mosaic virus (TMV) movement protein; apo, apple pectinase-apoplastidal targeting sequence; nos-T, *Agrobacterium tumefaciens* nopaline synthetase terminator; LB, left border; RB, right border. **(B)** Construct used for the expression of mouse ZP2 peptide. **(C)** Construct used for the expression of mouse Izumo1 peptide. TT, T-cell epitope of tetanus toxoid (aa residues 830–844); mZP2, T- and B-cell epitopes of mouse ZP2 (aa residues 121–139); mIzumo1, B-cell epitope of mouse Izumo1 (aa residues 163–183); Linker; (GGGGS)3 flexible linker; 6xHis, Hexa histidine tag. SEKDEL: ER retrieval peptide.

and restriction enzyme digestion) were inoculated into 5 mL of Luria–Bertani (LB) medium supplemented with 50 µg/mL kanamycin and 50 µg/mL rifampicin and incubated overnight at 28°C with shaking (220 rpm). To prepare the main bacterial culture, 2 mL of the *Agrobacterium* suspension was placed in 200 mL LB medium containing the same antibiotics and incubated overnight (220 rpm, 28°C). *Agrobacterium* cells were harvested at 4560 × *g* for 30 min and resuspended in the infiltration medium (10 mM 2-(N-morpholino) ethanesulfonic acid [pH 5.8], 10 mM $MgSO_4$, and 0.02% v/v Silwet Gold) to achieve a final OD_{600} of 0.15–0.2. Bacterial suspension was then vacuum-agroinfiltrated into the leaves of 6–8-week-old greenhouse-grown *N. benthamiana* plants as previously described (Nausch and Broer, 2017).

2.3 Total soluble protein extraction from *N. benthamiana* leaves

Agroinfiltrated *N. benthamiana* leaves were harvested at various days post-infiltration (dpi), frozen at −80 °C, lyophilized using a freeze dryer system (Alpha 1–4 LD; Martin Christ GmbH, Osterode, Germany), and pulverized using a mixer. Small-scale protein extraction was performed to evaluate the protein expression and accumulation. Total soluble protein (TSP) was extracted from 25 mg of lyophilized leaf material, homogenized in 500 µL cold protein extraction buffer (100 mM NaCl, 10 mM KCl, 6.5 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.2) plus complete protease inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany), using a Precellys 24 homogenizer (Bertin Instruments, France), and incubated for 30 min on ice. Clarified extract was obtained via repeated centrifugation at 15000 × *g* for 15 min at 4°C. Then, protein concentration of the supernatant was measured using the Pierce Coomassie Plus (Bradford) assay kit (Thermo Fisher Scientific) with bovine serum albumin (BSA) (Thermo Fisher Scientific) as the standard.

2.4 Enrichment of proteins using the Ni-NTA purification system

Histidine (His)-tagged recombinant mZP2-3 and mIzumo1-3 were partially purified via immobilized metal affinity chromatography (IMAC) using a Ni-chelating resin. Leaf extracts (in 50 mM NaH_2PO_4, 300 mM NaCl, 250 mM sucrose, 5 mM imidazole) were centrifuged at 16,000 × *g* and 4°C for 1 h. Supernatants were collected and loaded onto a column (Bio-Rad Laboratories, Hercules, California, USA) containing pre-equilibrated Nuvia IMAC Ni-charged resin (Bio-Rad Laboratories). The column was washed twice with the wash buffer containing 20 and 30 mM imidazole. Recombinant protein was eluted with an elution buffer containing 300 mM imidazole, and the elution fraction was concentrated and desalted using Vivaspin 20 centrifugal concentrators with a 10 kDa cut-off membrane (Sartorius AG, Germany). LC-MS analyses of partially purified mIzumo1-3 protein were performed as already described (Klähn et al., 2021).

2.5 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting analysis

Protein samples were denatured at 95°C for 5 min in a loading buffer (10% glycerin, 150 mM Tris [pH 6.8], 3% SDS, 1% β-mercaptoethanol, and 2.5% bromophenol blue) and subjected to 12% SDS-PAGE. The separated proteins were transferred onto a 0.45-µm Hybond ECL nitrocellulose membrane (GE Healthcare Europe GmbH, Freiburg, Germany) using a Mini Trans-Blot electrophoretic transfer cell (Bio-Rad) at 100 V for 30 min. The membrane was blocked with Tris-buffered saline with Tween 20 (TBST; 20 mM Tris, 150 mM NaCl, and 0.05% [v/v] Tween20, pH 7.6) containing 5% (w/v) non-fat milk powder at 20–22°C (room

temperature [RT]) for 2 h. After washing thrice with TBST, the membrane was probed with mouse monoclonal anti-His antibodies (Dianova, Hamburg, Germany) at a 1:1,000 dilution in Signal Boost ImmunoReaction Enhancer solution I (cat. No. 407207; Merck KgaA, Darmstadt, Germany) at RT for 2 h. After washing, the membrane was probed with horseradish peroxidase (HRP)-conjugated donkey anti-mouse antibodies (Dianova) at 1:10,000 dilution at RT for 1 h. After a final wash with TBS, signals were detected via incubation with ECL chemiluminescence reagent and subsequent exposure of the membrane to a Kodak Biomax light X-ray film (VWR; Darmstadt, Germany).

2.6 Mouse immunization

Animal experiments were approved by Landesamt für Landwirtschaft, Lebensmittelsicherheit und Fischerei (Mecklenburg-Vorpommern, Germany; approval No. 7221.3-1-071/20-2) and performed in accordance with the German animal protection regulations.

Two groups of female BALB/c mice (6–8-weeks-old, n = 10 per group) were used for the immunization study (BIOSERV Analytik; Rostock, Germany). Each animal in the treatment group was injected subcutaneously with a physical mixture of partially purified plant-produced mZP2-3 and mIzumo1-3, containing approximately 5 µg of mZP2 and mIzumo1 peptides, respectively. Control group received similarly prepared extracts from pICH31120-infiltrated leaves. Mice were immunized four times at three-week intervals. All doses contained 10% Polygen (MVP Lab) as an adjuvant. Serum samples were collected prior to primary immunization and 20 days after each immunization to measure the antibody levels. Female BALB/c mice were mated with proven fertile BALB/c males of a similar age four weeks after the final immunization. Males were removed after two weeks, and the females were allowed to litter. Fertility was defined as the mean number of pups born.

2.7 Determination of antibody levels using enzyme-linked immunosorbent assay (ELISA)

Specific IgG responses in sera were determined using ELISA. Partially purified mZP2-3 and mIzumo1-3 were used to coat a 96-well plate. After incubation at RT for 2 h, the plates were washed thrice with phosphate-buffered saline (PBS) containing 0.05% Tween 20 and blocked with PBS containing 1% (w/v) BSA for 1 h at RT. After washing with PBST, diluted sera (1:25,000 in PBS) were added to the wells and plates were incubated for 2 h at RT. After washing with PBST, the plates were incubated with HRP-conjugated donkey anti-mouse secondary antibodies (Dianova) at a dilution of 1:2000 in PBS at 37°C for 1 h. After washing, the wells were incubated with tetramethylbenzidine substrate for 10 min in the dark, reaction was stopped using 250 mM H_2SO_4, and the OD at 450 nm was measured using a plate reader (Bio Tek; Bad Friedrichshall, Germany).

2.8 Histological examination

On day 135 of the experiment, mice were sacrificed. To monitor the effect of immunization on follicular development, ovaries were collected, fixed in 4% paraformaldehyde, and embedded with paraffin. Ovaries were sectioned at 5 µm, stained with hematoxylin and eosin, and observed under a microscope.

2.9 Immunofluorescence assay

Ovarian sections of immunized BALB/c and unimmunized wild mice were obtained from BIOSERV Analytik (Rostock, Germany). Ovarian sections were deparaffinized and subjected to antigen retrieval using sodium citrate buffer (10 mM sodium citrate, 0.05% Tween 20, pH 6.0). Sections were blocked with 10% goat serum blocking solution (Life Technologies, Frederick, Maryland, USA) at RT for 1 h, washed with PBS, and incubated with 1X mouse-on-mouse IgG blocking solution (Invitrogen, Thermo Fisher Scientific) at RT for 1 h. Sections of unimmunized wild mice were incubated with a 1:20 dilution of serum samples from vaccinated BALB/c mice and kept at 4°C overnight. Sections of immunized BALB/c mice were incubated with a blocking solution (without the addition of any serum samples), washed, and incubated with 10 µg/mL fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG secondary antibody (Invitrogen, Thermo Fisher Scientific) at 37°C for 1.5 h. After washing, slides were mounted with the DABCO mounting medium (25 mg/mL DABCO, 90% glycerol, and 10% PBS; pH 8.5) and observed under a fluorescence microscope.

2.10 Statistical analysis

Results are presented as the mean ± standard deviation. Experimental data were analyzed using IBM SPSS statistical software version 27. One-way analysis of variance and Student's *t*-test were used to compare the means. Duncan's test was used as a *post-hoc* test to measure the specific differences between the means. Statistical significance was set at $p < 0.05$.

3 Results

3.1 Transient expression of recombinant mZP2 and mIzumo1 peptides in *N. benthamiana* leaves

To determine the transient expression of peptides mZP2 (TDVRYKDDMYHFFCPAIQA) and mIzumo1 (LDCGERH IEVHRSEDLVLDCL) in *N. benthamiana*, codon-optimized constructs were cloned separately into the expression vector, pICH31120, of the MagnICON system. Expression cassettes were introduced separately into *N. benthamiana* leaves via agroinfiltration. Constructs mZP2-3 and mIzumo1-3 contained three repeated copies of antigenic peptides (Figure 1). A His-tag was added for the detection and purification of proteins.

Infiltrated leaves were harvested over time, and the expression levels of mZP2-3 and mIzumo1-3 were determined using western blotting. A strong protein band with an expected size of approximately 13 kDa for monomeric proteins confirmed the expression of mZP2-3. Weak signals were detected at approximately 26 and 39 kDa, representing the dimeric and trimeric forms of the protein, respectively. Production of mZP2-3 was initiated on 4 dpi, increased until 8 dpi, and then decreased. In contrast, the control sample (pICH31120-infiltrated leaf extract) exhibited no signal (Figure 2A).

Plants infiltrated with the mIzumo1-3.1 construct, in which the His-tag was located in the C-terminal region, exhibited no protein band in the western blot using antibodies against His tag (Figure 2B). Therefore, a second construct (mIzumo1-3.2), with a His-tag at the N-terminus, was designed to express mIzumo1 in plants. Western blotting analysis of crude protein extracts from leaves infiltrated with mIzumo1-3.2 revealed a band of approximately 25 kDa corresponding to the dimer form of mIzumo1-3 protein (Figure 2C); therefore, mIzumo1-3.2 was used for subsequent experiments. The highest accumulation of mIzumo1-3 was detected on 5–6 dpi.

Infiltrated leaves developed a necrotic phenotype that was more severe in leaves expressing mIzumo1-3 than in those expressing mZP2-3 (Figure 2D). Optimal harvesting time points for mZP2-3 and mIzumo1-3 were determined to be 8 and 6 dpi, respectively, when the protein accumulation was high. Subsequently, severe leaf necrosis and wilting were observed along with a decrease in protein levels.

After protein extraction, mIzumo1-3 showed low stability in the extract. The stability of mIzumo1-3 protein was increased after the addition of protease inhibitors (Figure 3A) and storage at –80° C (Figure 3B).

3.2 Purification of plant-produced mZP2-3 and mIzumo1-3

Recombinant mZP2-3 and mIzumo1-3 were partially purified from the crude plant extracts under non-denaturing conditions using Ni^{2+}-charged column chromatography. Analysis of the partially purified mZP2-3 via western blotting revealed a dominant band of approximately 13 kDa and bands with molecular mass consistent with the monomeric and oligomeric forms of the antigenic construct, respectively (Figure 4A). In western blotting of the partially purified mIzumo1-3, a dominant band of approximately 25 kDa corresponding to the expected size of the dimer, and a band at the expected size of the tetramer form of mIzumo1-3 were detected, similar to those observed in the western blots of crude leaf extracts (Figure 4B). As the monomeric form of mIzumo1-3 was not detected via western blotting, a mass spectrometric analysis of the partially purified protein was performed, which confirmed the presence of mIzumo1-3 protein (Supplementary Material, Figure S1). ELISA revealed that the concentration of partially purified mZP2 was 29.3 µg/g dry weight (DW) and that of mIzumo1 was approximately 4.5 µg/gDW.

3.3 Immunogenicity of plant-expressed mZP2-3 and mIzumo1-3 in mice

To determine their immunogenicity, mZP2-3 and mIzumo1-3 were partially purified on a large scale and used in a prime-boost immunization assay. A group of 10 female BALB/c mice was immunized subcutaneously (four times at three-week intervals) with a mixture of equal amounts (5 µg) of plant-produced mZP2-3 and mIzumo1-3 antigens (Figure 5A). Mice in the negative

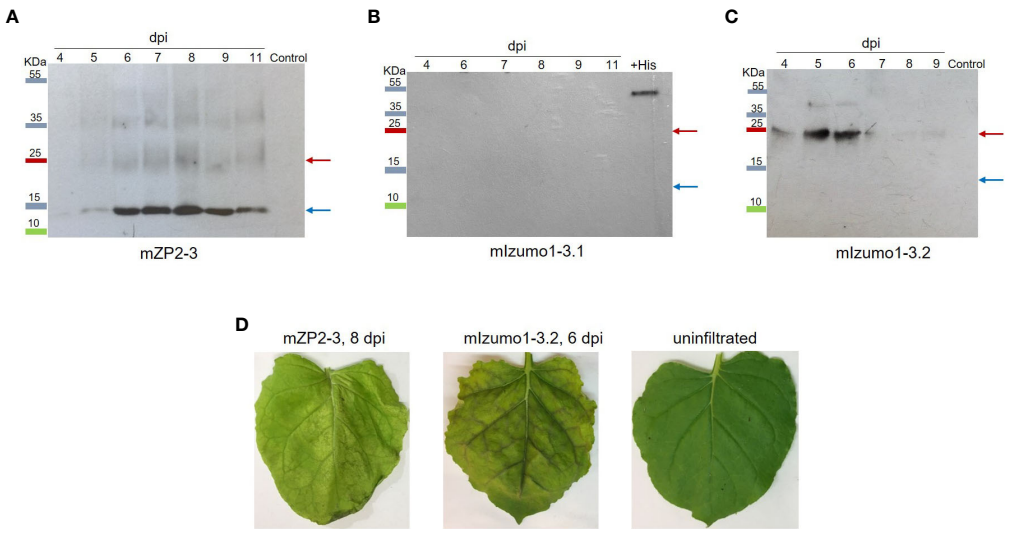

FIGURE 2
Expression of mZP2-3 and mIzumo1-3 in *Nicotiana benthamiana* leaves. Western blotting analysis of extracts from leaves infiltrated with mZP2-3 **(A)**, first mIzumo1-3 **(B)**, and second mIzumo1-3 **(C)** constructs at different dpi. Membranes were probed with a mouse anti-His antibody. Control, extract from pICH31120-infiltrated leaves; +His, a His-tagged protein as positive western blot control; dpi, days post-infiltration. Blue and red arrows indicate the expected size of the monomer and dimer forms of the proteins, respectively. **(D)** Phenotype of infiltrated leaves on optimal harvest day.

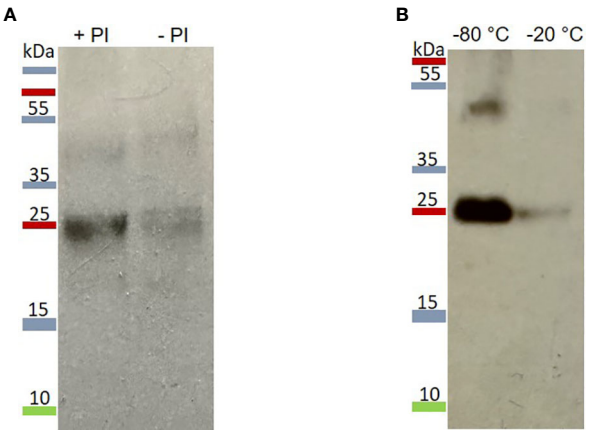

FIGURE 3
Stability of extracted mIzumo1-3. **(A)** mIzumo1-3 extracted from leaves with or without the addition of protease inhibitors. PI, protease inhibitor. **(B)** Detectable mIzumo1-3 after six weeks storage at −80 or −20°C. Membranes were probed with anti-His monoclonal antibodies.

control group were immunized with similarly prepared control plant extracts.

Humoral IgG antibody responses from individual mice were analyzed using ELISA at 1:25000 dilution of pre-immune and immune sera using plant-produced mZP2-3 or mIzumo1-3 as antigens. After primary immunization, no significant anti-mZP2-3 immune responses were detected in the sera of immunized mice compared to the pre-immune sera collected before primary immunization and that from the control group. However, second immunization induced an IgG antibody response that was significantly ($p < 0.0001$) higher than that in the primary dose and control group. Each subsequent immunization significantly increased the anti-mZP2-3 antibody response (Figure 5B).

Immunized mice exhibited a more potent anti-mIzumo1-3 antibody response than that observed at the pre-immune levels, even after primary vaccination with a physical mixture of recombinant proteins (Figure 5C). Immunization with the second and third doses significantly ($p < 0.0001$) increased the antibody levels against mIzumo1-3. Antibody response continued to increase after the last immunization; however, it was not significant compared with that after the third dose (Figure 5C). Although some absorption was detected in the serum samples of the control group, it was significantly ($p < 0.0001$) lower than that in the serum samples of the antigen-vaccinated group (Figures 5B, C).

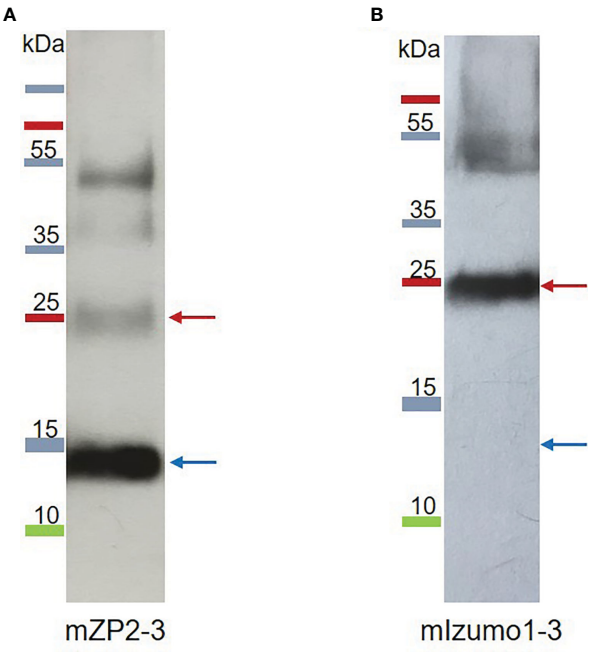

FIGURE 4
Western blotting analysis of partially purified plant-produced mZP2-3 **(A)**, and mIzumo1-3 **(B)** proteins. Proteins were purified from *N. benthamiana* leaves via Ni-NTA affinity chromatography, separated via sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and probed with anti-His monoclonal antibodies for western blotting. Blue and red arrows indicate the monomer and dimer forms of the proteins, respectively.

3.4 Effects of immunization on fertility and ovarian morphology

Immunized female mice were mated with proven fertile male mice three weeks after the final vaccination and allowed to produce litters to determine the effect of immunization on mouse fertility. Mice in the group treated with mZP2-3 and mIzumo1-3 showed a 39% reduction in average fertility compared to that in the control group. Average litter size was reduced from 8.4 ± 0.86 (mean ± standard error of the mean) in the control group to 5.1 ± 0.64 in treatment group immunized with mixture of plant-produced mZP2-3 and mIzumo1-3 antigens. Although the difference in mean litter size between the groups was not significant, the number of pups born correlated with the level of individual antibodies against both mZP2-3 and mIzumo1-3 proteins ($p < 0.05$; Figure 6). Moreover, negative correlation between litter size and total antibody levels (anti-mZP2-3 + anti-mIzumo1-3) in individual mice was significant ($p < 0.01$; Supplementary Material, Figure S2).

To determine whether immunization with recombinant antigens negatively impacts the ovary, mouse ovaries were isolated for observation. Immunization with plant-produced antigens caused no oophoritis or damage to the ovaries as they appeared normal. Additionally, histological analysis of ovarian sections from immunized mice did not reveal any abnormal follicular development or follicular degeneration. Figure 7 shows the normal follicles/oocytes at various stages of growth and development.

3.5 *In vivo* localization and *in vitro* binding of antibodies to native ZP

Immunofluorescence assay was used to determine the presence of induced antibodies bound to ZP in immunized mice. In ovaries collected from mice immunized with a physical mixture of plant-

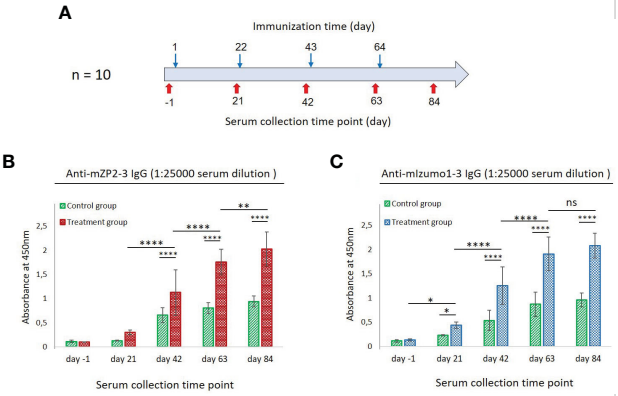

FIGURE 5
Antibody responses to mZP2-3 and mIzumo1-3 after subcutaneous immunization of mice. **(A)** Schematic representation of immunization and blood collection. Enzyme-linked immunosorbent assay (ELISA) for detection of anti-mZP2-3 **(B)** and anti-mIzumo1-3 **(C)** antibody responses in sera collected from the treatment (mice vaccinated with a mixture of plant-produced mZP2-3 and mIzumo1-3) and control (injected with the control plant extract) groups. Absorbance values are shown at 1:25,000 dilution of sera. Data are represented as mean ± standard deviation (SD). Absorbance was measured at 450 nm. *$p < 0.05$; **$p < 0.01$; ****$p < 0.0001$; ns, not significant.

produced mZP2-3 and mIzumo1-3, immunofluorescence was observed from ZP on the surface of oocytes, indicating that anti-mZP2 antibodies bind to ZP *in vivo*. Ovarian sections obtained from mice in the control group failed to show any signals from ZP (Figure 8A).

Immune serum samples obtained after the final injection of plant-produced antigens were used for their reactivity with the ovarian sections of wild mice in an indirect immunofluorescence assay. When serum antibodies produced in vaccinated BALB/c mice were applied to the ovarian sections from unvaccinated wild mice, fluorescence signals were observed around the oocyte, indicating the binding of antibodies to native ZP. Notably, ovarian sections treated with pre-immune serum did not show any antibody reactions with the ZP matrix (Figure 8B). Reactivity of the immune sera with spermatozoa was not evaluated as acrosome-reacted mouse sperms could not be obtained for this study.

4 Discussion

In this study, we aimed to investigate whether a combination of the small putative contraceptive peptides, mZP2 and mIzumo1, produced in *N. benthamiana* can reduce the fertility of immunized mice. Production of mouse-specific peptides is essential for their subsequent use as oral baits, which is the only practical way to immunize wild mouse populations. Plants are cost-effective and safe productions systems for the oral delivery of vaccines, without the need for any expensive purification steps (Daniell et al., 2009; Chan and Daniell, 2015). Moreover, plant-produced peptides can be bio-encapsulated in plant cells and protected from the gastro-intestinal environment that can limit the efficiency of free orally-delivered peptides (Kwon and Daniell, 2016; Pantazica et al., 2021). In addition, expression of contraceptive peptides in plants in fusion with highly stable proteins such as virus like particles can significantly improve their stability and immunogenicity to induce systemic and mucosal immunity in orally vaccinated mice (Ghasemian et al., 2023a).

Peptides were selected based on their immunogenicity, contraception capability (Sun, 1999; Naz, 2008; Wang et al., 2009), and potential mouse specificity (Hardy et al., 2008; Inoue et al., 2008; Xue et al., 2016). Previous studies have investigated the efficacies of synthetic peptides (Sun, 1999; Naz, 2008) or *Escherichia coli*-produced recombinant forms of peptides (Hardy et al., 2008; Wang et al., 2009) as contraceptive immunogens. Small peptides are generally unstable in plants (Kusnadi et al., 1997; Demain and Vaishnav, 2009). However, for the first time, we demonstrated the production of small peptides in sufficient amounts to induce antibody formation and reduce the fertility of BALB/c mice in this study. Moreover, we showed that the antibodies induced against plant-produced peptides can bind to wild-type mouse ovaries *in vitro*.

In accordance with our previous report that three repeats of mZP3 peptide significantly increased the antigen yield (Ghasemian et al., 2023b), the production of mZP2 and mIzumo1 in the form of three-peptide antigens resulted in sufficient amounts of the peptides in plants in this study. Our results confirmed the successful expression of the C-terminal His-tagged mZP2-3 construct. However, mIzumo1-3 protein was detectable using anti-His antibodies only when the His tag was placed at the N-terminus. Although localization of the His-tag is generally believed to have

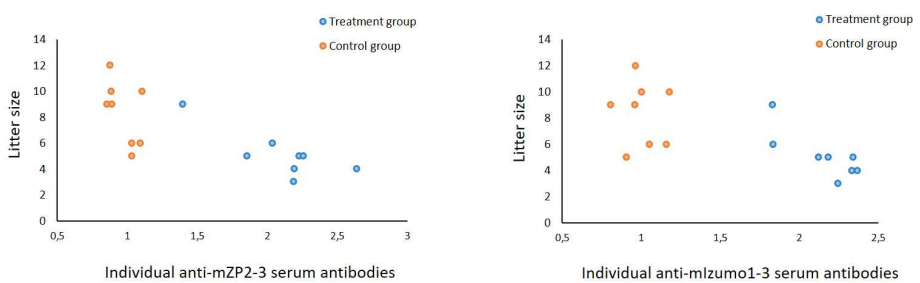

FIGURE 6
Correlation between anti-mZP2-3 and anti-mIzumo1-3 antibody levels and the litter size. Blue circles indicate IgG values in individuals in the group vaccinated with the mixture of mZP2-3 and mIzumo1-3. Orange circles indicate the IgG values in the control group. A significant negative correlation between the antibody levels and litter size was observed in the treatment group ($p < 0.05$).

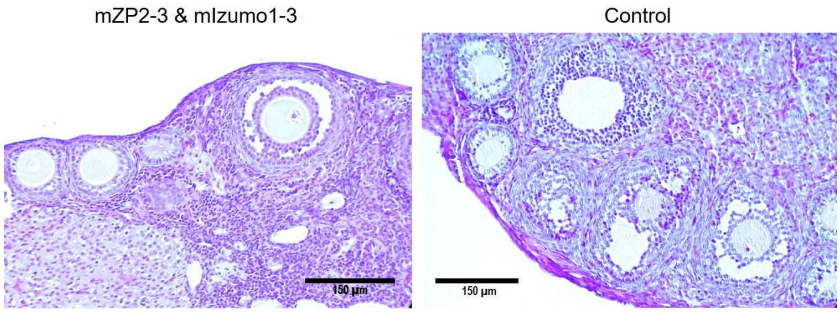

FIGURE 7
Ovarian histology of immunized BALB/c mice. Section from ovary of mouse immunized with the mixture of plant-produced mZP2-3 and mIzumo1-3 (left) exhibited normal growing follicles at various development stages similar to that from the control mouse immunized with the control plant extract (right). Ovarian sections were stained with hematoxylin and eosin. Scale bar: 150 µm.

little or no effect on the structure and function of proteins, some studies have reported that the His tag and its position affect protein properties, such as production and stability (Mohanty and Wiener, 2004; Booth et al., 2018; Bulaon et al., 2020). Although mZP2-3 was detected at a molecular mass expected for the monomeric form, mIzumo1-3 was detected as a dimer, similar to previous reports demonstrating the ability of Izumo1 to form dimers that are stable and can survive SDS heat treatment (Ellerman et al., 2009; Inoue et al., 2013; Inoue et al., 2015). Moreover, His tags can mimic protein interactions and allow the protein to form higher-order oligomers (Mohanty and Wiener, 2004; Majorek et al., 2014).

Accumulation of mIzumo1-3 was significantly lower than that of mZP2-3 in infiltrated leaves. In addition to the rate of protein synthesis, the rate of protein degradation is an important factor affecting the final accumulation of recombinant proteins (Benchabane et al., 2009; Egelkrout et al., 2012). Here, we observed severe necrosis in leaves expressing mIzumo1-3 compared to leaves expressing mZP2-3. In response to infiltration, plants induce hypersensitive necrosis, which further induces the expression of proteases degrading the recombinant proteins (Pillay et al., 2014; Nosaki et al., 2021). Hence, the low accumulation of mIzumo1-3 in this study may be the result of degradation by plant proteases. Moreover, mIzumo1-3 was not stable after extraction in the absence of protease inhibitors, indicating the sensitivity of mIzumo1-3 to proteases released from plant organelles into the extract (Doran, 2006; Benchabane et al., 2009). Therefore, further optimization is needed to increase the stability and yield of mIzumo1 peptide in plants.

As equal amounts of mZP2 and mIzumo1 peptides needed to be administered and the volume of vaccine that could be injected into mice was limited, restricted amounts of the plant-produced peptides (5 µg of each in the mixture) were used in the immunization study.

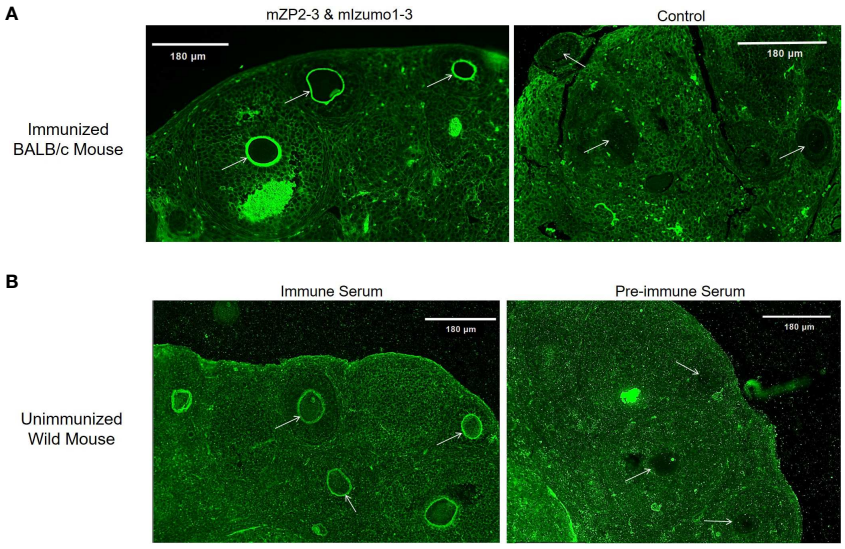

FIGURE 8
Reactivity of antibodies generated in immunized mice with zona pellucida (ZP). **(A)** *In vivo* binding of induced antibodies to the ovaries of immunized BALB/c mice. FITC-conjugated goat anti-mouse IgG detects induced antibodies bound to ZP in mice immunized with the physical mixture of plant-produced mZP2-3 and mIzumo1-3 (left), whereas no signal was observed on ZP of control mouse immunized with the control plant extract (right). **(B)** Indirect immunofluorescence assay for *in vitro* reactivity of BALB/c serum samples with wild mouse ZP. Ovarian sections were treated with 1:20 dilution of immune (left) and pre-immune (right) serum samples. Arrows indicate ZP. Scale bar: 180 µm.

Compared with individual component epitopes, immunization with a combination of epitopes from different reproductive proteins involved in various steps of fertilization improves the immunogenicity and efficacy of vaccines (Hardy et al., 2004; Hardy et al., 2008; Choudhury et al., 2009). Here, we demonstrated the abilities of plant-produced mZP2-3 and mIzumo1-3 to induce significant levels of antibodies in immunized mice. Antibodies against mZP2-3 and mIzumo1-3 were detected in all immunized mice, although there was variation in antibody responses among individual mice, consistent with other reports (Hardy et al., 2008; Naz, 2008). In previous studies, anti-mZP2 and anti-mIzumo1 antibodies have been detected in mice after immunization with 20 µg of bacterial-produced mZP2 peptide (Hardy et al., 2008) or 75 µg of synthetic mIzumo1 peptide (Naz, 2008). However, in this study, 5 µg of plant-produced mZP2 and mIzumo1 was sufficiently immunogenic in mice. This might indicate the contribution of the production system in enhancing the immunogenicity of antigens (Schellekens, 2002). The responses detected in control mice may be due to the presence of antibodies against plant proteins co-purified with plant-produced immunogens and also present in ELISA detecting antigen (Naupu et al., 2020).

In this study, after the administration of plant-produced antigens, a 39% decrease in the mean number of pups was observed, which correlated with the levels of antibodies against both mZP2-3 and mIzumo1-3 antigens. *In vivo* binding of antibodies to the oocyte was detected in immunized animals. It is assumed that antibodies induced against the mZP2 peptide reached the target sites around the oocytes, preventing the spermatozoa from reaching the oocyte and efficiently disrupting fertilization (Duckworth et al., 2007). Immunity to these contraceptive peptides did not cause histological ovarian disruption or follicular depletion. Therefore, the observed decrease in litter size of vaccinated mice cannot be attributed to ovarian atrophy, oophoritis, or loss of normal follicles (Koyama et al., 2005; Lloyd et al., 2010). Although the reactivity of anti-mIzumo1 antibodies with sperms was not evaluated in this study, a significant correlation was observed between anti-mIzumo1 antibody levels and the number of pups born. The negative correlation between individual antibody responses and litter size suggests that higher antibody responses may lead to greater reduction in the number of pups. Compared with other studies, in which a significant reduction in fertility was achieved with higher concentrations of mZP2 and mIzumo1 (Hardy et al., 2008; Naz, 2008), a low dose (5 µg) of plant-produced mZP2 and mIzumo1 was sufficient to reduce the fertility of mice in this study. Immunization of mice with different concentrations of mIzumo1 peptide decreases the fertility in a dose-dependent manner (Naz, 2008; Wang et al., 2009; Naz, 2014). High doses of antigens induce sufficient levels of antibodies to significantly increase the contraceptive effect of vaccines (Pomering et al., 2002; Naz, 2008; Wang et al., 2009). These studies suggest that higher concentrations of plant-produced mZP2 and mIzumo1 may cause a greater reduction in fertility.

Reactivity of antisera from immunized mice with wild mouse oocytes indicated that immunization with plant-produced mZP2 generates antibodies reactive with the native ZP of wild mice. Although wild mouse sperms were not available for antibody reactivity assays, the correlation of anti-mIzumo1-3 antibodies with litter size suggested the contribution of mIzumo1 to the reduced fertility of immunized mice. Based on these data, we can infer that plants producing sufficient amounts of mZP2 and mIzumo1-3 may effectively reduce the fertility of the wild mouse populations via immunocontraception.

To the best of our knowledge, this is the first report on the production of the small contraceptive peptides, mZP2 and mIzumo1, using a plant production system. Our results demonstrated that mZP2 and mIzumo1 peptides can be expressed in *N. benthamiana*. We found that a low dose of plant-produced peptide antigens was immunogenic in mice and induced significant immune responses. Moreover, we observed a reduction in the number of pups and a negative correlation between litter size and the level of antibody response in mice vaccinated with plant-produced antigens. However, as only one antigen dose and a limited number of vaccinated animals were used in this study, further studies should use increasing doses of plant-produced peptides to induce higher titers of antibodies and investigate their effects on mouse fertility. Nevertheless, the findings of this study can be used to develop effective oral contraceptive vaccines to manage mouse populations.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Ethics statement

The animal study was reviewed and approved by Landesamt für Landwirtschaft, Lebensmittelsicherheit und Fischerei (Mecklenburg-Vorpommern, Germany; approval No. 7221.3-1-071/20-2).

Author contributions

KG designed and performed the experiments, analyzed the data and wrote the manuscript. IB and JH supervised the work, gave scientific advice and revised the manuscript. JS supervised the histological and immunofluorescence studies and supported data interpretation. NK and RK carried out the animal experiment. SM performed mass spectrometric analyses. All authors contributed to the article and approved the manuscript.

Funding

The plant work was funded by the scholarship of the 'Landesgraduiertenförderung' of the University of Rostock.

Acknowledgments

The authors would like to acknowledge Dr. Udo Meyer and Susanne Montag, Bioserv GmbH Rostock, for their assistance during the animal trial. We are grateful to Patrick Inomoto and

Mahshid Monavari (Innocent Meat GmbH, Rostock, Germany) for kindly providing the fluorescence microscope and for their assistance. We are also grateful to Kerstin Thoss for technical assistance with ELISAs and Sonja Konopka for delivering the plants. We also thank Editage (www.editage.com) for English language editing.

Conflict of interest

Authors NK and RK were employed by the company BIOSERV Analytik und Medizinprodukte GmbH Rostock, Germany.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2023.1191640/full#supplementary-material

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Supplementary material to 2.3

MHHHHHHQYIKANSKFIGITELGGGGSGGGGSGGGGS**LDCGERHIEVHRSEDLVLDCL**GGGG
SGGGGSGGGGS**LDCGERHIEVHRSEDLVLDCL**GGGGSGGGGSGGGGS**LDCGERHIEVHRSED**
LVLDCLSEKDEL

Figure 2.3 S1: Amino acid sequence of mIzumo1-3 protein. Three peptides identified by LC-MS/MS are colored in (i) yellow, (ii) light orange and light blue, (iii) darker orange and darker blue. The three copies of the mIzumo1 peptide are shown in bold letters.

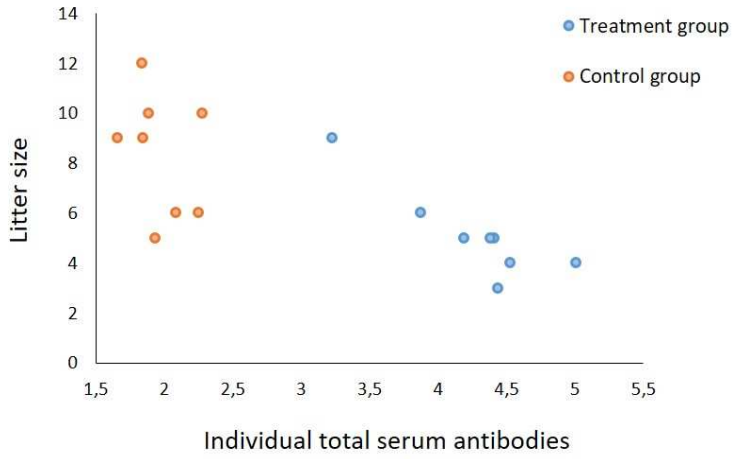

Figure 2.3 S2: Correlations between the total IgG (anti-mZP2-3 + anti-mIzumo1-3) antibody levels and the litter size. The blue circles indicate total IgG values from individuals in the group vaccinated with mixture of mZP2-3 and mIzumo1-3 and the orange circles indicate the values from control group. There was a significant negative correlation in the treatment group at the level of $p < 0.01$.

3. Discussion

To control the mouse populations in the field, an orally applicable mouse-specific contraceptive antigen is required. In this work, we describe the production of three putative mouse-specific contraceptive peptides in plant using a transient expression system based on vacuum agroinfiltration. This approach provides a rapid and simple way to investigate the production of recombinant proteins in plants. The results presented in this study reveal the possibilities, as well as the limitations, that come with the production of small peptides. However, some attempts at improving stability and concentration were successful. The immunogenicity and contraceptive efficacy of plant-produce peptides were investigated in the BALB/c mouse model. In addition, immunohistochemistry was applied to ovarian sections of wild mice to evaluate the reactivity of induced antibodies to native zona pellucida.

Already, contraception efficiency of these peptides has been investigated using bacterial-produced recombinant peptides or synthetic peptides (Hardy et al., 2002a; Hardy et al., 2008; Naz, 2008). However, production using plants could be more promising since plant production can be easily and inexpensively scaled up to produce safe and free-pathogen proteins (Egelkrout et al., 2012; Burnett and Burnett, 2020a). In the case of oral vaccine, which is the desired approach for targeting mouse populations, plants are the most promising candidates because the plant cell wall can protect the peptides from degradation in the acid stomach environment (Kwon and Daniell, 2015; Rosales-Mendoza and Nieto-Gómez, 2018).

3.1 Can small peptides be produced in plants?

For the first investigations on the production of small peptides in plants and the administration of isolated proteins in animal studies, we used the transient gene expression technology. Transient transformation using *A. tumefaciens* has been a useful procedure for rapid evaluation of protein expression, characterization, functional studies, and optimization of expression parameters compared to transgenic plants (Lico et al., 2008; Tremblay et al., 2010). *N. benthamiana* has been widely used as a host for transient expression procedure by leaf infiltration due to its ease and high efficiency, which is amenable to agroinfiltration and can rapidly produce high-yields of biomass (Goodin et al., 2008; Chen and Lai, 2013).

In a first attempt I tried to optimize the production of mZP3 peptide in plants using different constructs, mZP3-1, GFP-mZP3-1, mZP3-3, and HBcAg-mZP3. Based on these results the production of mZP2 and mIzumo1 peptides were performed using three-peptide antigens, mZP2-3 and mIzumo1-3. The intracellular localization of plant-produced mZP3 aimed to be

ER since mZP3 is naturally glycosylated (Chen et al., 1998; Avella et al., 2013). mZP2-3 and mIzumo1-3 were also targeted to the ER to increase the accumulation of recombinant proteins in plants (Karg and Kallio, 2009; Mandal et al., 2016).

Infiltration of the small peptide mZP3 (15 aa) in the form of a mono-peptide antigen (mZP3-1) into *N. benthamiana* resulted in low amounts of mZP3 compared to other constructs. The two main factors that impact the final accumulation of recombinant proteins are protein synthesis and degradation rates (Kusnadi et al., 1997; Benchabane et al., 2008; Egelkrout et al., 2012). mRNA of mZP3-1 was detected in leaves and transcript structure analysis predicted stem-loop structures (Fig. 3-1), suggesting that the transcription in plant or mRNA instability is probably not the reason for lower yield of mZP3-1 protein. The same seems to be true for the other proteins. The predicted secondary structure of the different mRNAs gives no reason to assume that the transcript is highly unstable. They all show only short unpaired regions and are folded into stem-loop structures that are supposed to support transcript stability (Kuhn et al., 2001). However, there are several examples of recombinant proteins in which mRNA transcript levels have no correlation to product accumulation (Richter et al., 2000; Doran, 2006). Low accumulation of products have been observed for several recombinant proteins, despite effective transcription, suggesting the influence of translation rate and protein degradation on overall accumulation (Rozkov et al., 2000; Benchabane et al., 2008). Since we directly optimized the codon usage to the plant host, limited translation is also not assumed to be the reason for the low protein content (Egelkrout et al., 2012; Habibi et al., 2017).

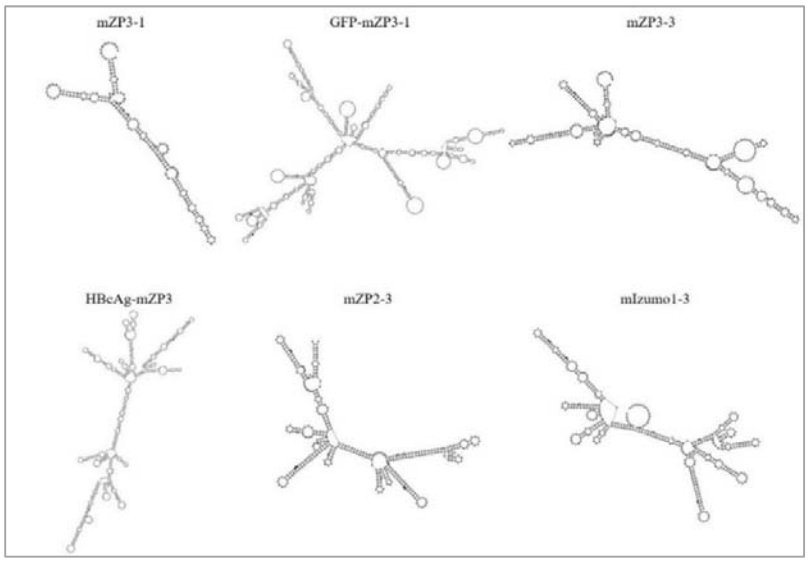

Figure 3.1: Predicted secondary structure of mRNAs corresponding to the plant-produced proteins.

Some post-transcriptional interventions such as degradation of foreign proteins are contributing factors that affect the protein yield (Rozkov et al., 2000; Egelkrout et al., 2012). Since mZP3-1 and mZP3-3 genes are very similar and the only difference is the number of epitope copies, the effective factor that leads to the higher stability and accumulation of the mZP3-3 might be the size. In general, it is believed that smaller peptide and protein sequences are degraded faster, thus reducing the level of the final product (Kusnadi et al., 1997; Demain and Vaishnav, 2009). Therefore, since increasing the antigen size by tripling of the mZP3 peptide resulted in a 25-fold accumulation of mZP3 in infiltrated leaves, the low amounts of mZP3-1 could be attributed to the small size of the protein and its susceptibility to proteolytic processes. This hypothesis is also supported by the GFP fusion. Nevertheless, GFP is a very stable protein and there are several reports that show that GFP fusion successfully improve production and stability of recombinant proteins in various conditions (Piron et al., 2014; Ponndorf et al., 2017; Polasa et al., 2022). On the other hand, although mZP2-3, mZP3-3, and mIzumo1-3 had approximately similar sizes, they showed different accumulation in plants. I discovered that mIzumo1-3 showed a low yield while mZP2-3 and mZP3-3 accumulation was significantly higher than that of mIzumo1-3. The difference in the accumulation of these proteins may be due to the difference in the sequence of their peptide components. The overall accumulation of the protein depends on characteristic, stability or susceptibility of peptides on the protein (Faye et al., 2005; Benchabane et al., 2008).

The other observation that suggests the protein degradation as the contributing factor in low accumulation of proteins is the massive early damage in leaves expressing mZP3-1 and mIzumo1-3. Hypersensitive necrosis responses are commonly induced in *N. benthamiana* in response to the *Agrobacterium* infiltration at different dpi, depends on the recombinant protein. This includes the induction of proteases that cause proteolytic degradation of recombinant proteins and reduce the final yield (Pillay et al., 2014; Zhou et al., 2017). Leaf necrosis has already been reported for many other recombinant proteins that showed prohibitive levels and resulted in low amounts of the target proteins (Nausch et al., 2012; Hamorsky et al., 2015). Both larger proteins, GFP-mZP3-1 and mZP3-3, which showed greater accumulation, reduced leaf necrosis compared to the small mZP3-1 protein, although the same bacterial strain and vector system were used in infiltration. These observations support the assumption that size is the effective factor in low accumulation of mZP3-1.

Early necrosis was also evident after infiltration with mIzumo1-3 compared to mZP2-3 and mZP3-3, despite similar sizes. It is hypothesized that the production of mIzumo1-3 may cause

more stress to plants and result in low yield. This effect could be attributed to the sequence of mIzumo1 peptide, since the other parts of the constructs were common in sequence. It is likely that the necrosis observed a few days after infiltration was too extensive and the living tissue was not sufficient to produce significant amounts of protein. Therefore, low levels of mIzumo1-3 and mZP3-1accumulation can also be the result of degradation by plant proteases (Kusnadi et al., 1997; Fischer and Emans, 2000). The proteolytic processes are induced following the hypersensitive necrosis responses and can degrade the recombinant proteins and limit the accumulation in plant expression systems (Benchabane et al., 2008; Singh et al., 2022). Furthermore, we observed that after crude protein isolation from *N. benthamiana* leaves, the stability of mIzumo1-3 was low and mZP3-1 was not even detectable. This instability may be associated with degradation by proteases released into the extract from the plant compartments. Some particular plant organelles such as vacuoles contain various proteases (Doran, 2006; Benchabane et al., 2008). These observations suggest the sensitivity of mIzumo1-3 and mZP3-1 to plant proteases. Any recombinant protein can contain susceptible amino acids accessible to proteases and degradation in different plant species (Doran, 2006; Benchabane et al., 2008).

In contrast to earlier observations, the accumulation of mZP3-3 was slightly higher compared to that of mZP2-3, although more phenotypic disorder was observed in leaves producing mZP3-3. It may indicate the higher stability of mZP3-3 in plants, which can be attributed to glycosylation of the protein. Glycosylation can enhance the overall stability of the protein and influence protein properties including resistance to proteolytic degradation (Faye et al., 2005; Benchabane et al., 2008). Instability is an issue with the small therapeutic proteins, and glycosylation is one of the promising approaches to increase protein stability. The glycans may enhance the overall stability of the protein by influencing its tolerance to proteolytic enzymes (Kusnadi et al., 1997; Demain and Vaishnav, 2009). Therefore, the higher stability of mZP3-3 might be due to glycosylation, which makes it more resistant to proteases caused by hypersensitive necrosis.

mZP3 was also produced in the form of mZP3-presenting HBcAg VLPs, as HBcAg is strong in provoking the IgG and IgA immune responses and are promising candidates for oral vaccine delivery (Holmgren and Czerkinsky, 2005; Berardi et al., 2020). HBcAg-mZP3 indicated a rapid and high production in *N. benthamiana*. VLPs could be successfully produced and assembled in plant expression systems with high speed and yield (Chen and Lai, 2013; He et al., 2021). HBcAg VLPs are stable under diverse physical stress conditions

(Strods et al., 2015; Schumacher et al., 2018) and increase the stability of HBcAg-fused protein compared to unfused protein (He et al., 2021). In this study, expression of HBcAg-mZP3 overcame the severe necrosis of the infiltrated leaves and resulted in significant increase in mZP3 yield compared to mZP3-1. Although major parts of HBcAg-mZP3 were insoluble, the level of mZP3 obtained from soluble HBcAg-mZP3 was similar to that of mZP3-3 protein and 25 times higher than that of mZP3-1. Insolubility of HBcAg has already been reported in production of recombinant cytosolic HBcAg as well (Peyret, 2015b; Pang et al., 2019).

Numerous antigens are incompatible with assembly of VLP, depending on factors such as the length and charge of the foreign antigen (Chen and Lai, 2013; Bayliss et al., 2020). However, we observed distribution of HBcAg-mZP3 in particular sucrose density fractions (Huang et al., 2006; Peyret, 2015a; Berardi et al., 2017) and formation of HBcAg-mZP3 particles similar to wild-type (Huang et al., 2006; Berardi et al., 2017) and chimeric HBcAg VLPs (Yang et al., 2017; Peyret et al., 2020) produced in plants. Therefore, presentation of mZP3 on HBcAg did not interfere with the assembly of VLPs and might lead to its high immunogenicity compared to HBcAg-free mZP3.

In this study, we targeted all recombinant proteins to the ER to minimize protein degradation (Benchabane et al., 2008; Desai et al., 2010). Moreover, mZP3 peptide is naturally a glycosylated peptide and its glycosylation influences the efficiency. Contraceptive antibody responses to mZP3 were induced in the mice against glycosylated epitopes more than against un-glycosylated peptide (Hardy et al., 2003). Glycosylation of the plant-produced mZP3 peptide could be determined according to the protein size and reaction with concanavalin A, a carbohydrate-binding protein that binds to the sugar residues of proteins (mainly mannosyl and glucosyl groups) (Bierhuizen et al., 1988; Chakravarty et al., 2005). The carbohydrate binding ability of the lectin concanavalin A can only visualize the attachment of carbohydrates to glycoproteins and does not give any information concerning the composition of the glycans, nevertheless it is sufficient to show that glycosylation occurs. The attachment of glycans to a polypeptide affects properties of a protein, including resistance to denaturation and protection from proteolytic degradation. It can also improve biological functions such as immunogenicity (Wandelt et al., 1992; Lindh et al., 2009; Rosenberg et al., 2022). Therefore, localization of mZP3 in the ER through conjunction with an ER retention signal can enhance its accumulation and stability in plants. We may assume the higher stability of mZP3-3, compared to similar size proteins mZP2-3 and mIzumo1-3, could be due to the glycosylation. Production of mZP2

and mIzumo1 was also targeted to the ER although they are not glycosylated. Nevertheless, since we did not test the cytosol or other organelles to target the proteins, we are not able to explicitly claim an influence of ER targeting on our proteins stability and accumulation. More approaches to improve protein expression, stability, and accumulation in plants can be considered in further studies.

Our results showed successful production of all three mouse-specific peptides, mZP2, mZP3, and mIzumo1 in *N. benthamiana* plants. To create an effective vaccine, it is necessary to produce a sufficient amount of stable antigen. We could achieve sufficient amount of peptides by modifying the expression constructs and using a fusion strategy. Host species, transformation protocol and subcellular localization are other effective parameters in the optimal yield of recombinant proteins (Benchabane et al., 2009; Burnett and Burnett, 2020a), which can be considered in further investigations to enhance accumulation of plant-produced contraceptive peptides. However, we can conclude that plants are able to produce small mouse-specific peptides in sufficient amounts to allow a vaccination.

3.2 Can plant-produced ZP and sperm peptides induce immune responses in mice?

3.2.1 Subcutaneous immunization

Contraceptive vaccines efficacy is associated with induction of specific antibodies against proteins or peptides involved in the reproduction process (Naz, 2005; Gupta, 2015). The role of antibody response in mediating immunocontraception has been shown by inhibiting the attachment of sperm to oocyte (Lloyd et al., 2010; Gupta, 2022). In this study, we examined the ability of plant-produced peptides to induce specific antibodies in immunized mice. Since the immunization and mating studies are problematic in wild mice, studies were carried out using the laboratory BALB/c mice and partially verified with sections of wild mice ovaries.

GFP-mZP3-1 induced serum IgG against mZP3 peptide after the first boost, while mZP3-3 induced antibodies in some mice already after priming and led to significantly higher immune responses. It indicates the higher immunogenicity of multi-peptide antigen compared to a single-peptide antigen (Hardy et al., 2004; Redwood et al., 2007). Although mZP2 and mIzumo1 peptides were not used as single-peptide antigens in immunization study, the immunogenicity of only 5 µg/dose of them as mZP2-3 and mIzumo1-3 was demonstrated in subcutaneously immunized mice. Notably, both peptides were immunogenic in relatively low amounts, compared to previous reports (Hardy et al., 2008; Naz, 2008). Successful immunogenicity of such low doses might be due to using a physical mixture of mZP2-3 and

mIzumo1-3 antigens in immunization of mice (Gupta et al., 2003; Hardy et al., 2008; Naz, 2008). Different studies represented the efficiency of combination of synthetic or recombinant peptides or proteins containing various epitopes of ZP and sperm-proteins over individual components. The simultaneous induction of antibodies against sperm and ovary proteins seems to increase the effectiveness of the vaccine (Lea et al., 2002; Hardy et al., 2008; Choudhury et al., 2009; Arukha et al., 2016). Hence, it might be assumed that this is also the case in our study. Nevertheless, since the single vaccines were not tested it remains to be ascertained whether this holds true.

Immunogenicity of 50 µg mZP3 in the form of mZP3-3 was higher than 5µg mZP2 and mIzumo1 in the form of mZP2-3 and mIzumo1-3, respectively (Table 3.1), which could be attributed to the higher epitope dose or glycosylation of mZP3 that can influence the immunogenicity (Gomord and Faye, 2004; Bosch and Schots, 2010). Despite the equal doses of glycosylated mZP3, anti-mZP3 antibody titers in response to mZP3-3 were higher compared to those against GFP-mZP3-1, which can be attributed to repeated copies of mZP3 peptide in the mZP3-3 antigen (Redwood et al., 2007; Hardy et al., 2008). mZP2-3 and mIzumo1-3 which contain the same number of peptides and were used in equal doses in a vaccine mixture, showed comparable antibody titers after the final immunization. Hence, the immunogenicity of mZP2 and mIzumo1 epitopes appears to be similar in mice. Therefore, in addition to epitope dose, the number of epitopes in the antigen and the immunogenicity of individual epitopes affect the immunogenicity of the antigen.

Table 3.1: Summary of the injected antigens and levels of the induced antibodies

Plant-produced Antigen	Peptide number in the Antigen	Peptide Dose	Detected Antibody	Antibody Level (mean ± SD absorbance at 10^5 dilution)
GFP-mZP3-1	1	50 µg	Anti-mZP3	0.57 ± 0.18
mZP3-3	3	50 µg	Anti-mZP3	1.04 ± 0.28
HBcAg-mZP3	1	5.5 µg	Anti-mZP3	0.98 ± 0.3
mZP2-3	3	5 µg	Anti-mZP2-3	0.6 ± 0.11
mIzumo1-3	3	5 µg	Anti-mIzumo1-3	0.64 ± 0.13

Unlike mZP3-3, the injected HBcAg-mZP3 contained mZP3 at a dose similar to the injected mZP2 and mIzumo1 peptides. Nevertheless, immunogenicity of 5.5 µg/dose mZP3 presented on HBcAg VLPs was significantly higher than those of 5 µg/dose mZP2 and mIzumo1 in the

form of multi-peptide antigens. One reason may be the higher immunogenicity of mZP3 compared to the other two peptides. Previously, immunization of mice with an antigen containing the same number of mZP3 and mZP2 epitopes resulted in the induction of higher antibody titers against mZP3 than that of mZP2 (Hardy et al., 2008). The other reason can be vaccine synergy with VLPs as an immunogenic carrier (Kushnir et al., 2012; Diamos et al., 2019). The use of chimeric HBcAg VLPs is one of the powerful strategies to improve the immunogenicity of inserted small molecules (Ulrich et al., 1998; Pumpens and Grens, 2001; Grgacic and Anderson, 2006). High immunogenicity of HBcAg mZP3 might also be attributed to the presence of several helper T-cell epitopes within HBcAg and to its polymeric structure (Francis et al., 1990; Noad and Roy, 2003; Grgacic and Anderson, 2006). Inserting the foreign epitopes into the major immunodominant region (MIR) of HBcAg present the insertions on the surface of particles (Brown et al., 1991; Pumpens and Grens, 2001). It seems that insertion of mZP3 into MIR facilitated the presentation of mZP3 to the immune system and resulted in high immunogenicity of mZP3. We observed that immunization with 5.5 µg/dose mZP3 in the form of HBcAg-mZP3 elicited anti-mZP3 antibody titers comparable to those induced by 50 µg/dose of mZP3 in the form of mZP3-3. It seems that the density and accessibility of mZP3 epitope, due to its frequent presentation on the particles surface, enhance immune responses against this heterologous epitope. Therefore, lower doses of VLP-presented epitopes than free epitopes are usually sufficient to obtain comparable antibody titers (Noad and Roy, 2003). In addition to the effect of antigen structure, the difference in immunogenicity can also be attributed to the epitope dosage. There is a relationship between vaccine dose and effectiveness, however, a higher dose is not necessarily the optimal dose (Pniewski et al., 2011; Benest et al., 2022). It is possible that too much vaccine can cause the absence of normal immune responses, while low doses may be sufficient (Daniell et al., 2019). Therefore, low dose of mZP3 in HBcAg-mZP3 may be closer to optimal dose and a reason for its higher immunogenicity compared to mZP3-3. However, the optimal dose varies between vaccines and should be determined by experiments.

Comparing the vaccine dose and antibody formation induced by the plant made ZP and Izumo peptides with similar peptides either produced synthetically or in bacteria, it may be assumed that the plant production system is superior (Table 3.2). Contribution of production systems in immunogenicity of antigens has already been assumed (Schellekens 2002). For mZP3 this can be attributed to the glycosylation that does not occur in bacteria (Hardy et al. 2003). Plant-produced mZP2 and mIzumo1 may differ from the synthetic or bacterial peptides due to other

plant-specific modifications of the proteins, which can affect the performance of the recombinant products and influence the immunogenicity (Faye et al. 2005; Streatfield and Howard 2003; Bosch and Schots 2010). However, since the production systems and antigens are different and such low doses have not been tested in other studies, we cannot explicitly claim about the most immunogenic peptides.

Table 3.2: Contraceptive peptides used as vaccine in this and previous studies

Antigen	Peptide	Peptide Dose	Antibody	Reference
E. coli-produced polyepitope Antigen	mZP3	20 µg	Lack of antibody responses	(Hardy et al., 2004)
Plant-produced mZP3-based antigens		5.5 & 50 µg	Significant antibody responses (at serum dilutions greater than 1: 10^5)	(Ghasemian et al., 2023a,b)
E. coli-produced polyepitope Antigen	mZP2	20 µg	Significant antibody responses (at serum dilutions greater than 1:100)	(Hardy et al., 2008)
Synthetic mZP2 peptide		100 µg	Significant antibody responses (at 1:4×10^4 serum dilution)	(Sun, 1999)
Plant-produced mZP2-3		5 µg	Significant antibody responses (at serum dilutions greater than 1:10^5)	(Ghasemian et al., 2023c)
Synthetic mIzumo1 peptides	mIzumo1	75 µg	Significant antibody responses (at 1:50 serum dilution)	(Naz, 2008)
Plant-produced mIzumo1-3		5 µg	Significant antibody responses (at serum dilutions greater than 1:10^5)	(Ghasemian et al., 2023c)

It can be concluded that all plant-produced peptides, mZP3, mZP2 and mIzumo1, are capable of inducing systemic antibody responses through subcutaneous injection. However, immunogenic epitope, epitope dose, and vaccine components are important factors in inducing adequate immune responses. Nevertheless, HBcAg-mZP3 was the most potent immunogen compared to other antigens, therefore, plants producing HBcAg-mZP3 VLPs were orally administered to mice.

3.2.2 Oral immunization

Oral delivery of leaf tissues expressing HBcAg-mZP3 resulted in humoral and mucosal immunity. Plant-produced HBcAg-mZP3, without the addition of adjuvants, was able to induce IgG and IgA antibody responses against mZP3. The compact and highly ordered structure of VLPs can enable them to tolerate the harsh intestinal environment to stimulate strong immune responses (Huang et al., 2005; Berardi et al., 2017; Berardi et al., 2020). In addition to stable structure of VLPs, T-cell antigenic epitopes within the HBcAg can act as

immune stimulating adjuvants (Chen and Lai, 2013; Diamos et al., 2019). Moreover, bio-encapsulation of antigens in plant cells as well as adjuvant effects of plant components could optimize the effect of oral antigens (Rosales-Mendoza and Salazar-González, 2014; Kwon and Daniell, 2016).

Our results support the use of a highly stable and immunogenic partner such as VLPs as carriers of small peptides for resistance in the digestive system and induction of immunity. Induction of immunity was not as rapid and high as that in parenteral vaccination. Immunogenicity of antigens depends on the conditions of their application and administration route (Lobaina et al., 2003; Pyrski et al., 2019). Oral administration of vaccines may result in reduced immunogenicity and require larger amounts or multiple doses of antigens compared to those administered by injection (Streatfield and Howard, 2003). Nevertheless, we concluded that the plant-produced mZP3 presented by HBcAg VLPs are orally immunogenic and induce specific mucosal and systemic immune responses in mice. However, an effective regime for adequate function of vaccine must be determined.

3.3 Does immunization with plant-produced peptides affect fertility?

Despite the immune responses against mZP3, there was no correlation between anti-mZP3 antibodies and reduction of fertility in this study. Moreover, neither IgG nor IgA anti-mZP3 antibodies specifically bound to oocytes in BALB/c mice *in vitro*, whereas both antibodies recognized the native ZP in wild mice specifically. Since the sequence of mZP3 is conserved between the two strains, we hypothesized that in BALB/c mice, ZP3 is not accessible to antibodies or induced antibodies are not cross-reactive to the target (Frank et al., 2005) in BALB/c. Hardy et al. (2002) have also reported that under identical conditions, antibodies against mZP3 successfully reduced fertility in wild but not BALB/c mice (Hardy et al., 2002a). The level of contraception induced by mouse ZP3 peptides depends on mouse strains (Hardy et al., 2003; Hardy et al., 2004). Therefore, it might be possible that plant-produced mZP3 is able to induce contraception in wild mice. This expectation gains further support by the results of immunohistochemistry analysis showing the reactivity of anti-mZP3 antibodies to wild mouse ZP.

In contrast to anti-mZP3 antibodies, anti-mZP2 induced antibodies bound to ZP in immunized BALB/c mice. The observation indicated the cross-reactivity and localization of antibodies to the target site in the ovarian ZP, which can inhibit sperm-egg binding and leads to the reduced fertility (Sun, 1999; Duckworth et al., 2007). Fertility of mice immunized with the mixture of mZP2-3 and mIzumo1-3 was reduced. A 39% reduction in the mean litter size was observed in

the group treated with plant-produced antigens compared to that of the control group. Moreover, antisera from immunized BALB/c mice reacted with wild mouse ovarian ZP *in vitro*. These findings indicate that anti-mZP2 antibodies are cross-reactive antibodies and their targets are accessible in both BALB/c and wild mice. According to *in vitro* data, it can be hypothesized that the antibodies against plant-produced mZP2 may react to wild mice ZP *in vivo* and reduce fertility too. Although the reactivity of anti-mIzumo1 antibodies to mouse sperms could not be evaluated in this study, the correlation of litter size with anti-mIzumo1-3 antibody levels indicates the contribution of anti-mIzumo1 antibodies in reduced fertility.

In contrast to some reports (Borillo et al., 2008; Calongos et al., 2009; Lloyd et al., 2010), autoimmunization of mice with mZP2 and mZP3 peptides in this study did not cause ovarian dysfunction or oophoritis. These observations suggest the safety of plant-produced peptides and support the conclusion that reduction in fertility of immunized mice was due to induced antibodies and not to ovarian dysfunction.

The correlation between the litter number and antibodies against both mZP2-3 and mIzumo1-3 raises the possibility that significant reduction in fertility and number of pups might be achieved by inducing higher antibody responses. Since previous studies have reported that antibody generation and fertility inhibition of mZP2 and mIzumo1 peptides are dose-dependent (Naz, 2008; Wang et al., 2009), higher doses of epitopes may result in higher levels of antibody responses, leading to greater contraception efficacy. It has been expected that a vaccine with 50–70% efficacy will be effective in wildlife populations control (Naz, 2005). The observed infertility in mice immunized with plant-produced mZP2 and mIzumo1 and the reactivity of anti-mZP3 antibodies with wild mouse ZP are promising findings that indicate the potential of plant-produced peptides to achieve the desired infertility effect.

3.4 Conclusion

The results of this study warrant the use of plants as a system for the production and delivery of mouse-specific oral immunocontraceptive vaccines and suggest that their efficacy be investigated in wild mice. We observed that including the repeated epitopes, combination of different epitopes, and fusion to an immunogenic stable carrier such as VLPs increase the efficiency of peptide-based antigens. Hence, it is assumed that the development of a polyepitope vaccine that simultaneously targets mZP2, mZP3, and mIzumo1 peptides could be promising for the induction of effective contraception. Moreover, presentation of the polyepitope antigen on VLPs can support both immunogenicity and stability in oral rout. Therefore, contraceptive effect of a plant-produced vaccine containing mZP2, mZP3, and

mIzumo1 repeats presented on VLPs needs to be investigated as an oral vaccine in wild mice or other mouse strains. The work presented in this thesis can make significant contribution to the development of oral immunocontraceptives as a significant tool to control populations of wildlife. We must keep in mind that 100% contraceptive effectiveness is not required nor desirable in wildlife population control.

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5. Appendix

5.1 List of figures

5.2 List of Tables

5.3 Acknowledgements

This PhD project would not have been completed without the great help and supports of many people whom I would like to acknowledge and express my sincere gratitude to.

First and foremost, I deeply appreciate and thank my supervisor, Prof. Dr. Inge Broer, for her perfect guidance and scientific vision, valuable advices and ideas, and great support and patience which helped me to overcome many difficulties during the study.

My sincere thanks are extended to Dr. Jana Huckauf who has been always available to respond my questions and offer constructive suggestion and comments. Special thanks go to Ms. Kerstin Thoß and Ms. Sonja Konopka for their great help and technical assistance. I would like also to express my great gratitude to Prof. Julian Ma and Prof. Dirk Prüfer who accepted to review and examine this thesis.

I really appreciate the collaboration and help of Dr. Henrik Nausch. I also want to thank all former and current members of Agrobiotechnology Department at University of Rostock, Dr. Axel Masloboy, Mr. Patrick Inomoto, Mr. Jakob-Maximilian Keller, Ms. Katharina Unkel, Dr. Daniel Ponndorf, Ms. Sarah Treibing, Ms. Kathrin Johnsen, Ms. Denise Heß, Ms. Jennifer Steinke, and Ms. Hsuan-Wu Hou.

Lastly and most importantly, I offer my immense appreciation and gratitude to my dear husband, parents and siblings for helping me get through the difficult times by blessings, sacrifices, and constant love and support. I am forever indebted to you.

5.4 Authors contribution

The manuscripts included in the thesis and my contribution to these works are described below.

Ghasemian, K.; Broer, I.; Schön, J.; Kolp, N.; Killisch, R.; Huckauf, J. Plant-Produced Mouse-Specific Zona Pellucida 3 Peptide Induces Immune Responses in Mice. *Vaccines* 2023, 11(1), 153.

Author's contribution: I designed and cloned the used constructs and performed all experiments such as agroinfiltration, DNA, RNA and protein analyses. I prepared the material for animal studies and analyzed the data. I wrote the manuscript.

Co-authors: I.B. and J.H. supervised the work, gave scientific advice, and revised the manuscript. J.S. supervised the histological and immunofluorescence studies. N.K. and R.K. performed the animal experiment.

Ghasemian, K.; Broer, I.; Schön, J.; Killisch, R.; Kolp, N.; Springer, A.; Huckauf, J. Oral and Subcutaneous Immunization with a Plant-Produced Mouse-Specific Zona Pellucida 3 Peptide Presented on Hepatitis B Core Antigen Virus-like Particles. *Vaccines* 2023, 11(2), 462.

Author's contribution: I designed and cloned the constructs, performed all plant experiments and the molecular analysis. I performed the statistical analysis and wrote the manuscript.

Co-authors: I.B. and J.H. supervised the study, provided scientific advice, and revised the manuscript. J.S. supervised the histological and immunofluorescence analyses. R.K. and N.K. performed animal experiments. A.S. performed electron microscopy.

Ghasemian, K.; Broer, I.; Schön, J.; Kolp, N.; Killisch, R.; Mikkat, S.; Huckauf, J. Immunogenicity and contraceptive efficacy of plant-produced putative mouse-specific contraceptive peptides. *Front. Plant Sci*. 2023, 14:1191640.

Author's contribution: I designed the constructs, carried out the plant and molecular experiments, conducted the analysis and wrote the manuscript.

Co-authors: IB and JH supervised the work and revised the manuscript. JS supervised the histological and immunofluorescence studies and supported data interpretation. NK and RK carried out the animal experiment. SM performed mass spectrometric analyses.

5.5 Curriculum vitae

Personal Information

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Education

April 2016 – Present	Doctoral candidate at Agrobiotechnology, Faculty of Agricultural and Environmental Sciences. University of Rostock, Germany.
2009 – 2012	Master of Science in Agricultural Biotechnology. Department of Agricultural biotechnology, Bu-Ali Sina University, Hamedan, Iran. GPA: 18.55 / 20 (A).
2004 – 2009	Bachelor of Science in Agricultural Engineering-Plant Protection. Department of Plant Protection, Shiraz University, Shiraz, Iran. GPA: 17.41 / 20 (A).
2003 – 2004	Pre-University Course Education in Field of Experimental Science, Sadat-Rafei Pre-University Center, Shiraz, Iran.

Publications

Khadijeh Ghasemian, Inge Broer, Jennifer Schön, Nadine Kolp, Richard Killisch and Jana Huckauf (2023). Plant-Produced Mouse-Specific Zona Pellucida 3 Peptide Induces Immune Responses in Mice. Vaccines, 11(1), 153.

Khadijeh Ghasemian, Inge Broer, Jennifer Schön, Richard Killisch, Nadine Kolp, Armin Springer and Jana Huckauf (2023). Oral and Subcutaneous Immunization with a Plant-Produced Mouse-Specific Zona Pellucida 3 Peptide Presented on Hepatitis B Core Antigen Virus-like Particles (2023). Vaccines, 11(2), 462.

Khadijeh Ghasemian, Inge Broer, Jennifer Schön, Nadine Kolp, Richard Killisch, Stefan Mikkat and Jana Huckauf (2023). Immunogenicity and contraceptive efficacy of plant-produced putative mouse-specific contraceptive peptides. Front. Plant Sci., 14:1191640.

Khadijeh Ghasemian, Sonbol Nazeri, Abdolkarim Chehregani Rad and Asghar Mirzaie Asl (2015). Producing of embryogenic callus from zygotic embryo in *Dorema ammoniacum* D. Journal of Plant Research, 28(2): 364-373.

Khadijeh Ghasemian, Sonbol Nazeri, Abdolkarim Chehregani Rad and Asghar Mirzaie Asl (2012). Stages of somatic embryogenesis using zygotic embryo in *Dorema ammoniacum* D. Cell and Tissue Journal, 3(1): 21-27.

Conference publications

2022	Meeting of the International Society for Plant Molecular Farming in Rome, Italy. Flash talk and poster presentation: Immunization of mice with the plant-made mice-specific contraceptive ZP3 peptide stimulates antibody responses.
2019	International Plant Science Conference in Rostock, Germany. Talk with the topic: Plant-made peptides as immunocontraceptives.
2018	Meeting of the International Society for Plant Molecular Farming in Helsinki, Finland. Poster presentation: Plant-made putative rodent-specific contraceptive small ZP3-peptide.
2018	Young Scientist Meeting in Braunschweig, Germany. Talk with the topic: Plant-made putative contraceptive peptides
2017	Molecular Breeding Workshop in Braunschweig, Germany. Poster presentation: Expression of putative rodent-specific contraceptive small peptides in *Nicotiana benthamiana*.
2012	National Conference of Sustainable Agriculture, Tehran, Iran. Poster presentation: Somatic embryogenesis from *Dorema ammoniacum* Zygotic embryos.
2011	7th National Biotechnology congress of Iran. Poster presentation: Evalution of Antimicrobial activity of *Mentha aquatic* Hydroalcholic extract.

Professional Experience

2018 – Present	Scientific coworker at the professorship for Agrobiotechnology, agricultural- and environmental faculty, University of Rostock, Germany.
2013	Plant cell and tissue culture teaching at University of Applied Sciences, Shiraz, Iran.
2011 – 2014	Part-time Research Assistant of Dr. S. Nazeri. (Faculty member of Biotechnology Department, Bu-Ali Sina University) in Plant cell and tissue culture. Bu-Ali Sina University. Hamedan, Iran.

Personal membership

Member of the international society for plant molecular farming (ISPMF)

Member of Society for Plant Biotechnology (Germany)

Member of Genetics Society of Iran

Research Experience and Interests

Gene cloning and transformation by *Agrobacterium tumefaciens*

Transient and stable transformation of plants

Analysis of DNA, RNA, and recombinant proteins

Immunohistochemistry analysis

Somatic embryogenesis

Cell and tissue culture of different medicinal plants and plant regeneration

Hairy root induction by *Agrobacterium rhizogenes*

Antibacterial activity assay

Antioxidant activity evaluation (Determination of phenol and flavonoid contents).

Languages

Persian	First language
English	Advanced
German	Intermediate

5.6 Declaration of independence / Eigenständigkeitserklärung

I declare that I have written the presented doctoral dissertation independently and without the assistance of others. I did not use any other sources, figures or resources than those indicated in the references. The present thesis has never been submitted for review in the present or similar version either abroad or in Germany.

Ich erkläre, dass ich die vorgelegte Dissertation selbstständig und ohne fremde Hilfe verfasst habe. Ich habe keine anderen Quellen und Hilfsmittel als die angegebenen benutzt. Die vorgelegte Dissertation wurde weder im Ausland noch im Inland in gleicher oder ähnlicher Form zur Prüfung vorgelegt.

Rostock, 28.06.2023

Khadijeh Ghasemian