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Two Zona Pellucida Vaccines Expressed in Different Plant Expression Systems for Spaying Mammals

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II. Index

I. Acknowledgement 1
II. Index 2
III. Figure index 4
IV. Table index VI
1. Abbreviations 8
2. Introduction 10
3. Material & Methods 20
3.1 Vector construction and cloning 20
3.2 Stable transformation & cultivation 21
3.3 Transient tobacco transformation 22
3.4 Protein isolation 22
3.5 Ni-NTA purification 23
3.6 Enzyme-linked immunosorbent assay (ELISA) 24
3.7 Western blot analysis 24
3.8 Degradation assay 25
3.9 Densitometric CTB determination 25
3.10 Northern blot analysis 25
3.11 Animal trial 25
3.12 Statistics 26
4. Results 27
4.1 Gene design and expression strategy 27
4.2 Vector construction 31
4.3 VacZP2 40
4.4 VacZP3 48
5. Discussion 54
5.1 Expression systems 54
5.2 VacZP2 61
5.3 VacZP3-P 66
5.4 Comparison between VacZP2 and VacZP3-P 69
5.5 CTB – A critical point in the method 70

5.6 Aims archieved ... 70

6. Summary ... 72

7. Reference ... 73

V. Supplement ... 95

VI. Authors contribution ... 113

VII. Declaration of independence ... 113

VIII. Publikationen und Tagungsbeiträge ... 114

IX. Curriculum vitae ... 115

III. Figure index

Figure 1: (A) Postulated ZP filament structure from chicken (adopted from Han et al. 2010). 11

Figure 2: Schematic illustration of the immunocontraception mechanism induced by zona pellucida proteins. 12

Figure 3: Milled sample from pea cotyledons using a mini-drill. 23

Figure 4: Vectors for VacZP2 expression. 29

Figure 5: Vectors for VacZP3 expression. 30

Figure 6: Overview of cloning strategies. 31

Figure 7: Verification of transforming *E. coli* with p35S-VacZP2. 31

Figure 8: RAPD-PCR with daf9 primer on p35S-VacZP2 and pSingle35S transformed *A. tumefaciens* clones. 32

Figure 9: Verification of transforming *E. coli* with pARC-VacZP2. 32

Figure 10: Colony-PCR of pICH-VacZP2 transformed (A) *E. coli* TG1 clones and (B) *A. tumefaciens* ICF320. 33

Figure 11: Restriction of pICH-VacZP2 from *E. coli* with *Eco*RI 33

Figure 12: Overview of cloning steps to pICH-$_{\Delta}$ZP2$_{only}$P. 34

Figure 13: Verification of transforming *E. coli* with pICH-VacZP2. 34

Figure 14: Cloning *vacZP3-P* into pJET1.2 35

Figure 15: Verification of transforming *E. coli* with pUC57 + VacZP3$_{BsaI}$ and pJET + VacZP3-P. 35

Figure 16: Restriction of pJET1.2 + VacZP3-P 36

Figure 17: Colony-PCR of pARC- VacZP3-P transformed *E. coli* TG1. 36

Figure 18: Overview of cloning strategies for VacZP3-P. 37

Figure 19: Colony-PCR for pICH-VacZP3-P 37

Figure 20: Overview of cloning steps to pET22b + VacZP3-B. 38

Figure 21: Colony-PCR with primers pJET1.2_fw + pJET1.2_rev of *E. coli* TG1 transformed with pJET1.2 + VacZP3-B. 38

Figure 22: Colony-PCR of *E. coli* TG1 transformed with pET22b + VacZP3-B. 39

Figure 23: Leaves of *N. benthamiana* infiltrated with *A. tumefaciens* ICF320 carrying (A) pICH-VacZP2 and (B) empty pICH29912 vector, ... 41

Figure 24: CTB concentration in different expression systems in relation to total soluble protein (TSP; dark green) and dry weight (DW; light green) 41

Figure 25: Stability of VacZP2 during storage in plant tissue. 42

Figure 26: Accumulation of VacZP2 in *N. benthamiana* plants after agrobacterium-mediated transformation of pICH-VacZP2 42

Figure 27: Detection of glycosylated VacZP2. 43

Figure 28: Stability of VacZP2 in tobacco leaf extracts over time. 44

Figure 29: Detection of $_{\Delta}$pZP2$_{only}$P after expression via MagnICON. 45

Figure 30: (A) Detection of antibodies against VacZP2 in FvB/NcrL mice. 46

Figure 31: (A) Detection of anti-VacZP2 and anti-$_{\Delta}$pZP2$_{only}$P antibodies after vaccination of golden hamster with 20 µg VacZP2 + 10 % Polygen. 47

Figure 32: Phenotypical changes of pICH-VacZP3 infiltrated *N. benthamiana* leaves after 5 and 10 days post infiltration (dpi). 48

Figure 33: Accumulation of VacZP3-P in different plant expressions systems measured by anti-CTB ELISA. 49

Figure 34: Stability of VacZP3-P during storage in plant tissue. 50

Figure 35: Detection of VacZP3-P via Western Blot with different primary antibodies. 50

Figure 36: Stability of VacZP3. 52

Figure 37: Antibody titer of vaccinated female BALB/c mice 53

Figure 38: Milestones in this concept. 71

IV. Table index

Table 1: Overview of ZP composition in different mammals 10

Table 2: Nomenclature of ZP Proteins. 12

Table 3: Overview of expression levels in tobacco leaves, seeds and carrot cells. 40

Table 4: Mating studies with vaccinated BALB/c mice. 53

Table 5: PCR program 1 95

Table 6: PCR program 2 „Dream60“ 95

Table 7: PCR program 3 „Colony-PCR“ 95

Table 8: PCR program 4 95

Table 9: List of used primer 96

1. Abbreviations

A

A.t.	agrobacterium tumefaciens
ANOVA	analysis of variance

C

CaMV	cauliflower mosaic virus

D

DNA	deoxyribonucleic acid

E

ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
ER	endoplasmatic reticulum

G

GCD	glucocerebrosidase
GFP	green fluorescent protein
GM1	monosialotetrahexosylganglioside
GSH	glutathione
GSSG	glutathione disulfide

H

HPR	horseradish peroxidase

I

IPTG	isopropyl β D 1-thiogalactopyranoside

K

KLH	keyhole limpet hemocyanin

L

LTB	heat labile enterotoxin B

M

MHC	major histocompatibility complex
M_r	relative molecular mass

N

NTA	nitrilotriacetic acid

P

PBS	phosphate buffered saline
PCR	polymerase chain reaction
PVS	perivitelline space
PZP	vaccine gained from pig oocytes containing whole porcine zona pellucida

R

RADP	randomly amplified polymorphic
RdRP	RNA-dependent RNA polymerase

RNA	ribonucleic acid
RT	room temperature

S

scFv	single-chain variable fragment
SPLPS	sodium phthalyted lipopolysaccharide

T

TBS	tris buffered saline
TBST	tris buffered saline tween 20, tris buffered saline
TMV	tobacco mosaic virus
TSP	total soluble protein
TVCV	turnip vein clearing virus

Z

ZP	Zona Pellucida

2. Introduction

Structure and function of the zona pellucida

The zona pellucida (ZP) is acellular glycoproteinmatrix, which surrounds the oocytes of all mammals (Chiu et al. 2014). Depending on the mammalian species (Table 1), the ZP is 7 - 20 µm thick and composed of 3-4 glycopro-teins which is designated as ZP glycoprotein-1 (ZP1), ZP2, ZP3 and ZP4 (Gupta et al. 2012). ZP is responsible for sperm-egg contact and thereby for fertilization. ZP1 and ZP4 are paralogous genes and share a common ancestor whereas ZP2 and ZP3 are relevant for ZP development and gamete recognition (Conner and Hughes 2003; Spargo 2002). Mice don't have a ZP4 in their egg coat, but they carry the gene (Goudet et al. 2008). The same accounts for bovine and canine ZP, but here it is ZP1, which is not integrated. The basic structure of the egg surface has been conserved over millions of years (Monné et al. 2006) and homologous genes to ZP1-4 building up the vitelline envelope of non-mammalian vertebrates and marine invertebrates, like mollusks and ascidians (Monné and Jovine 2011). For instance, humane ZP2 has an amino acid similarity of 57, 64, 94 % to murine, porcine and bonnet monkey ZP2, but humane ZP3 has 67, 74, 94 % similarity to murine, porcine and bonnet monkey ZP3, respectively (Chiu et al. 2014). Binding between sperm's ZP receptors at the acrosome membrane and ZP's sperm receptors initiate acrosome reaction and due to structural ZP changes, referred to as zona hardening, block polyspermy (Nixon et al. 2007). Despite of decades of research, the exact mechanism of fertilization is not totally revealed (Monné and Jovine 2011; Wassarman et al. 2001).

Table 1: Overview of ZP composition in different mammals (Goudet et al. 2008). Points (●) in brackets indicate pseudogene, which are not translated. ZP1 and ZP4 are paralogous.

Species	ZP1	ZP2	ZP3	ZP4
Pig		●	●	●
Dog	(●)	●	●	●
Cow	(●)	●	●	●
Cat		●	●	●
Rabbit		●	●	●
Human	●	●	●	●
Mouse	●	●	●	(●)
Rat	●	●	●	●
Hamster	●	●	●	●
Bonnet monkey	●	●	●	●

It is reported (Wassarman 1999; Greve and Wassarman 1985) that ZP1 acts as interconnection between ZP2/ZP3 heterocomplexes and builds up a filamentous structure (Figure 1A). Sperm binds to ZP, although ZP1 is lacking after mutation (Rankin et al. 1999). ZP2 and ZP3 are critical for the ZP structure. This was shown with ZP2 and ZP3 null mice, which produced no ZP and were infertile (Rankin et al. 2001; Rankin et al. 1998).

The sperm binds to ZP2 or ZP3 and penetrates the ZP by kinetic force of the flagellum or/and enzymatic reaction (Saldívar-Hernández et al. 2015) (Figure 1C). After reaching the perivitelline space (PVS) gametes membranes fuse and enzymes from exocytosed granules block the binding of a second sperm.

Due to the blocking of the contact between sperm and egg by mutating ZP2 (Sun 1999; Hasegawa et al. 1988; East et al. 1984) or ZP3 (Litscher et al. 2009; Kinloch et al. 1995; Millar et al. 1989), it still remains controversial that, which of the both ZP proteins is the actual receptor recognizing sperm contact (Dean 2004) (Figure 1B). It seems important in term of the taxon-specific fertilization. The swapping and site-directed mutagenesis in the murine *mZP3* exon-7 (Kinloch et al. 1995) governing the species-specificity in sperm-egg interaction (Swanson et al. 2001). Avella et al. 2014 supported the ZP2 model. Here, human sperm binds only to murine ZP, when a human ZP2 epitope (amino acid 55 - 88) was integrate into murine ZP2 by genetic modifications. Although it could be assumed, that a total distinction of species specific fertilization is not based on the molecular mechanism at all which is supported by the literature (Sinowatz et al. 2001). In some cases, sperm is able to bind different species (Lanzendorf et al. 1992; Yoshimatsu et al. 1988; Swenson and Dunbar 1982; Bedford 1977). The morphology of the sexual organs and times of fecundity or mating rituals are only some factors defining the reproduction of the species. When these barriers are artificially broken down, then it could be possible to create a new living creature, like the hinny or liger (Rong et al. 1988).

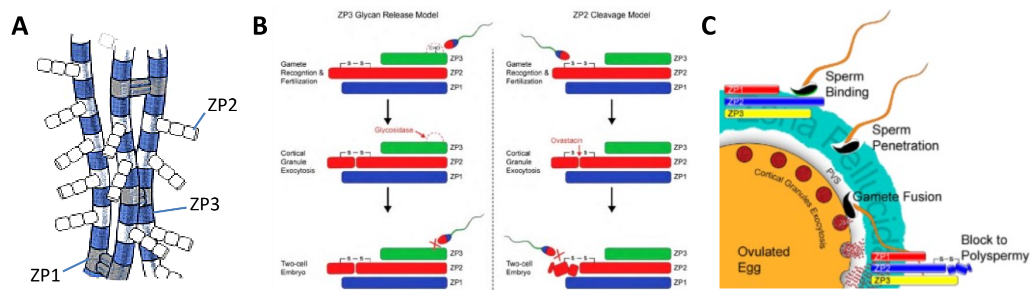


Figure 1: (A) Postulated ZP filament structure from chicken (adopted from Han et al. 2010). This imagination of ZP structure is similar to that of mice ZP. (B) Models of gamete recognition (Avella et al. 2013). When ZP3 is assumed as primary sperm receptor (left side), gamete binding is mediated by *O*-glycosylation at the *C*-terminus. Polyspermy is blocked by enzymatic digestion of the *O*-glycans. ZP2 as primary sperm binding site at the *N*-terminus (right side) (adopted from Avella et al. 2014). Enzymatic restriction destroys sperm binding site and disable second sperm binding. (C) Model of gamete recognition (adopted from Avella et al. 2014). Sperm bind to ZP2 at the surface of ZP (aquamarine). After reaching the perivitelline space (PVS) gametes membrane get fussed and polyspermy is blocked by degradation of the sperm binding site.

The nomenclature of ZP proteins in different mammals is complex (Harris et al. 2009; Conner et al. 2005). In this study, the ZP proteins are numbered from ZP1 to ZP4. This nomenclature is based on the molecular weight (Spargo 2002). For instance, ZP1 has the highest and ZP3 the lowest. ZP4 is paralogous gene of ZP1. The relationship to other ZP proteins is displayed in Table 2.

Table 2: Nomenclature of ZP Proteins. [1] Skinner et al. 1996; Harris et al. 2009; Spargo 2002. [2] Wardrip and Hedrick 1985; Yurewicz et al. 1987. [3] Dunbar et al. 1985; Harris et al. 2009. [4] Hasegawa et al. 1994.

Modern nomenclature [1]		Pig [2]	Pig [3]	Pig [4]	Mouse	Rabbit
ZP1	ZPB1	-	-	-	ZP1	-
ZP2	ZPA	ZP1/M_r 82,000/90K (= 65K + 25K)	ZP III	ZP1 (= ZP2 + ZP4)	ZP2	rc75
ZP3	ZPC	ZP3β	ZP Ia	ZP3β	ZP3	rc45
ZP4	ZPB2	ZP3α	ZP II	ZP3α	-	rc55

The research group of Isojima / Koyama / Hasegawa (Hyogo, Japan) named the *N*-terminus of porcine ZP2 (Ile_{36} – Ala_{168}, P42099, uniprot.org) "ZP4" and the complete porcine ZP2 were designated as "ZP1".

Immunocontraception

Since 1970, it is reported , that immunization with ZP proteins can reduce the fertility dramatically (Gwatkin et al. 1977; Sacco and Shivers 1973; Ownby and Shivers 1972; Shivers et al. 1972), without reacting on other tissues than the female reproduction organ (Palm et al. 1979). Two mechanisms could prevent fertilization after ZP vaccination (Figure 2). First, a humoral immune response, were ZP antibodies sterically mask sperm receptor and block contact between sperm and egg (Clydesdale et al. 2004; Barber and Fayrer-Hosken 2000; Bagavant et al. 1993). This contraception last as long as antibody titer drops. Second, a cellular immune response, which leads to degeneration of the follicle pool or prohibits their development (Lloyd et al. 2010; Li et al. 2008; Curtis et al. 2007; Koyama et al. 2005; Lou and Tung 1993; Skinner et al. 1984). This reaction is permanent, because the number of follicles is finite.

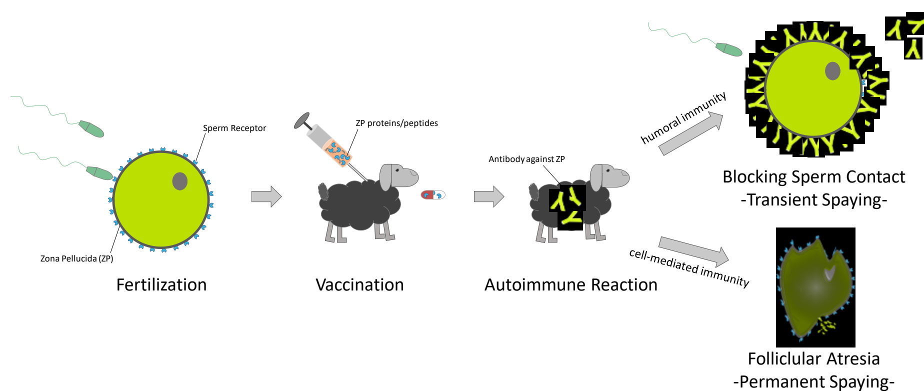


Figure 2: Schematic illustration of the immunocontraception mechanism induced by zona pellucida proteins. Vaccination with ZP can block sperm binding via antibodies or degradation of the follicles by macrophages.

Whether a humoral or a cellular response is triggered could be managed by immune stimulants, like adjuvants (Brunner et al. 2010), cytokines or interleukins (Li et al. 2007). The immune stimulants support either transient or permanent spaying (Figure 2). A study

with porcine ZP3 in marmosets shows a follicular atresia with complete Freund's adjuvants (CFA), but no atresia with sodium phthalyted lipopolysaccharide (SPLPS) (Upadhyay 1989).

Even if a ZP antigen is able to prevent fertilization in one species or race, the success could vary due to the individual MHC genotype (MHC polymorphism) (Fitchen et al. 1995). That means not all individuals respond to the ZP immunization with a complete contraception, which is a drawback in the development of an immunocontraceptive for individual birth control (Gupta et al. 2014; Bagavant 1997). The response could also be a reduced offspring. For regulating populations of wild animals, not all individuals have to respond after ZP immunization (Naz and Saver 2016; Kirkpatrick et al. 2011; Chambers et al. 1997; Knipling and McGuire 1972). It is also enough to lower the fertility to a threshold, where birth and death rate is at least equilibrated. This threshold is species-specific and for wild mice a reduction of fertility of 60 % is deemed from experimental and modelled data (Hardy et al. 2002b; Chambers et al. 1997).

Feral horses (Turner Jr. et al. 1997; Kirkpatrick et al. 1990; Liu et al. 1989), white-tailed deers (Naugle et al. 2002; McShea et al. 1997), elks (Shideler et al. 2001), bison (Duncan et al. 2013), grey seals (Brown et al. 1997) and elephants (Delsink et al. 2006) were regulated by immunizing with ZP proteins purified from pig ovaries (PZP). Porcine ZPs show the highest grade of cross reactivity among mammals, especially to ungulates (Frank et al. 2005), because of evolutionary conserved sequences. For example, 92.4 – 100 % contraception was achieved in a herd of wild horses in Maryland, USA over the time of 13 years (Kirkpatrick and Turner 2008). Another study with captive animals shows a mean spaying effect of 93.8 % of 30 mammalian species (Frank et al. 2005). ZP-immunocontraception is a promising alternative to hunting, trapping or poisoning e.g. in reservation zones or urban areas is forbidden, socially unpopular or inhuman (Kirkpatrick et al. 2011; Barfield et al. 2006; Grandy and Rutberg 2001; Oogjes 1997).

Recombinant ZP vaccines

The limitation of porcine oocytes, the relatively high costs per dosis (30 $, treatment excluded) and a potential to cause inflammations due to the contamination with oocyte-associated proteins which could push the development of recombinant vaccines (Naz and Saver 2016; Gupta et al. 2014; Kirkpatrick et al. 2011; Gupta 1997). Most of the them are composed of one of the four ZP proteins of various species and are expressed in *E. coli* (Gupta et al. 2013; Kitchener et al. 2009b; Lee et al. 1993), yeast (Lai 2004), insect cells (Hardy et al. 2003; Prasad et al. 1995) or mammalian cells (Clydesdale et al. 2004; Martinez 2000; Tsubamoto et al. 1999; Paterson et al. 1998). The *E. coli* expressed bonnet monkey ZP2 dramatically reduced the fertility in bonnet monkeys (Govind et al. 2002). In New Zealand Koalas (*Phascolarctos cinereus*) (Kitchener et al. 2009b) and Eastern Grey Kangaroos (*Marcopus giganteus*) (Kitchener et al. 2009a) were regulated by immunization with recombinant brushtrail possum (*Trichosurus vulpecula*) ZP3. Whereas, in most of the cases PZP was shown to be much more immunogenic than recombinant peptides or proteins (Miller et al. 2013).

To reach the market, recombinant ZP vaccines should feature a single application, long lasting protection and a new vaccine delivery system (Gupta and Minhas 2017; Gupta et al. 2011). Formulation for more effective delivery and strong adjuvant for immune

stimulation are the key factors in this development, by using just as antigen design (Gupta et al. 2011). Live bacteria (Zhang 1997) and viruses (Lloyd et al. 2007; Jackson 1998) were used to express ZP antigens directly in the animal and reduce population size very cheaply, but caused safety concerns for humans and other non-target species (Angulo and Cooke 2002). Another delivery approach is to entrap the antigen inside organic (e.g. lipid-based) or inorganic (polymer beads) particles to enable an ongoing vaccine release after dart gun injection (Garside et al. 2014; Gupta et al. 2011; Kanchan et al. 2009). Edible plant parts could be used for oral immunization delivered by a transmucosal carrier (Kwon and Daniell 2016). Mucosal vaccination using a bait could be an promising approach (Naz 2005).

Plant expression systems

Genetically modified plants can be used as bioreactors to produce recombinant proteins or chemicals for medicinal and commercial purposes, like therapeutic products, nutritional components, vaccine antigens, biodegradable plastics and industrial products (Sharma and Sharma 2009). This strategy is called molecular farming (Franken et al. 1997). Human growth factor expressed 1986 in transgenic tobacco was the first important pharmaceutical (Ma et al. 2003; Barta et al. 1986). Many other relevant pharmaceuticals like therapeutic proteins (Shaaltiel et al. 2007), antibodies (Cox et al. 2006) and vaccines (Mason et al. 1992) were produced in different plant expression systems (Yusibov et al. 2014). Using plants for production has some advantages, but also associated with disadvantages which are described in the following paragraph.

Advantages and disadvantages

Recombinant expression of biopharmaceuticals in plants is generally cost effective, because it does not require expensive fermenters, culture media (Chen and Davis 2016) or skilled personnel for production process (Twyman et al. 2003) and uses well established processes of crop cultivation and storage (Giddings 2001). Depending on the yield, it is estimated that recombinant proteins can be produced in plants at 2 – 10 % of the cost of microbial fermentation systems and at 0.1 % of the cost of mammalian cell cultures (Twyman et al. 2003; Giddings 2001). Downstream processing accounts for the most of the cost of the production (Obembe et al. 2011; Twyman et al. 2003; Evangelista et al. 1998) and when a high purity is required, all recombinant protein expression systems have similar cost of production (Twyman et al. 2003).

The production in plants is advantageous, when the pharmaceutical can be used in partly processed or unprocessed form, which could save the cost of downstream processing (Twyman et al. 2003). For example, the hepatitis b surface antigen produced in transgenic tobacco elicited a similar antibody response after injection as the commercial equivalent from yeast, but without expensive downstream processing by Ni-NTA chromatography (Thanavala et al. 1995). Saving in costs increases once more, when unpurified pharmaceuticals can be used without formulation, e.g. oral vaccines (Topp et al. 2016; Xiao et al. 2016) Plant material of the production host plant can be used for encapsulation of the vaccine on one hand and can boost the immune response on the other hand (Bosch and Schots 2010). The costs for a refreshment polio protection was dramatically reduced by a plant-based, “bioencapsulated”, oral polio vaccine (Daniell et al. 2016).

A premise for no or minimal purification of the pharmaceutical is lack of animal pathogens. Since animal pathogens not hosting in plants or are able to propagate (Buyel

and Fischer 2012; Commandeur et al. 2003; Streatfield and Howard 2003), this biosafety aspect is given, but no contaminations with mycotoxins, pesticides, herbicides and harmful endogenous plant secondary metabolites must be ensured (Doran 2000).

A further advantage of the plant-based production is the easy and fast scalability process, which reacts faster on market demand (Davies 2010). The production capacity can be modulated by using more or less land (Twyman et al. 2003). Furthermore stable and relatively cheap storage of recombinant proteins in plant tissue enables a cold-chain free and long-lasting storage (Twyman et al. 2005; Walmsley et al. 2003). Producing pharmaceuticals on the field includes concerns of horizontal gene transfer to other crops and wild-type plants. The isolation of the transgenes could be realized by geographical isolation, buffer plants, appropriate biological containment, like maternal inheritance or male sterility, and cultivation in a glasshouse (Daniell et al. 2009; Rigano and Walmsley 2005). Although cultivation in a glasshouse may limit the scalability and increases costs, it offers stable growing conditions and high containment (Mikschofsky and Broer 2012; Ma et al. 2003) and might comply regulatory requirements (Stöger et al. 2002).

Another benefit of the plant expression system is the glycosylation. Compared to yeast and insects, plant *N*-Glycosylation is much more similar to mammalian glycosylation, nevertheless plants add specific xylose and fucose residues that are not present in animals (Loos and Steinkellner 2014; Faye et al. 2005b). This fact could increase immunogenicity (Bosch and Schots 2010; Matoba et al. 2009; Bardor et al. 2003a; van Ree and Aalberse 1999; Kurosaka et al. 1991), but involves an individual testing of the plant-derived pharmaceutical to allergic reactions (Bardor et al. 2003a). Although post-translational modifications are slightly different, protein synthesis pathway is conserved between plants and animals, which makes plant-made mammalian proteins appear to fold and assemble correctly (Twyman et al. 2003).

Choice of production platform

To the high effective production of a pharmaceutical an appropriate plant transformation strategy (stable nuclear, cell suspension, transient expression system) and the suitable plant host is important (Obembe et al. 2011). These two factors determine together the accumulation level, the scalability, the storability, the costs and how the medication is administrated.

Stable nuclear transformation includes the integration of the transgene in the plant genome, which is inherited to the next generations. When used in whole land plants, it provide the highest scalability and lowest production cost in comparison to the other plant transformation strategies (Xu et al. 2012). This strategy accumulates the recombinant protein depending from the promoter in all plant cells or in specific tissues, like seeds. The CaMV35S promoter is known as a strong constitutive promoter (Seternes et al. 2016; Odell et al. 1985) and is often chosen for high expression in plants (Twyman et al. 2003). The finite shelf life of the tissue, like leaves and cell suspension makes a subsequent processing mandatory (Xu et al. 2012), but after freeze-drying many biopharmaceuticals show a high stability for months (Chan and Daniell 2015). The seed-specific expression in transgenic plants can be achieved by the *arcelin5*-I promoter from *Phaseolus vulgaris* (Goossens et al. 1994). Although CaMV35S expresses the transcript in leaves and seeds, CaMV35S seems to have a lower activity in seeds than in

leaves (Boothe et al. 2010). The use of a seed-specific promoter may improve the accumulation level (Jaeger et al. 2002; Fiedler et al. 1997). According to van Droogenbroeck et al. 2007 only 0.003 – 0.005 g *Arabidopsis* seeds are required to gain 100 µg scFv-Fc, expressed under the control of the phaseolin promoter (Sengupta-Gopalan et al. 1985). With the unknown seed protein (USP) (Bassüner et al. 1988) and the legumin B4 (leB4) (Bäumlein et al. 1986) promoter Scheller et al. 2006 expressed 0.5 % of TSP transgenic scFv antibody in transgenic tobacco seeds and increased this amount 40-times by fusing C-terminally 100 iterations of the elastin like binding (ELP) domains. The reason for seed-based production is the advantages over leaf-based production. Plant seeds are designed for the synthesis and long-term storage of proteins, which may account for 8 – 40 % of their weight (Hernández et al. 2013). With this feature, they are able to store heterologous proteins in high concentration in a small volume without further processing until use (Twyman et al. 2005; Stöger et al. 2005). In case of purification a reduced number of residing proteins in seeds, could lower the cost for fewer processing steps (Twyman et al. 2005; Jaeger et al. 2002).

Transient expression system mediated by a plant binary vector initiates the expression of a viral replicon and allows rapid protein production within a few days (Xu et al. 2012). Several plant viruses are used, like tobacco mosaic virus (TMV), cowpea mosaic virus (CPMV), potato virus (PVX), alfalfa mosaic virus and plum pox virus (Takeyama et al. 2015). This is typically carried out in *N. benthamiana* leaves, but it was also shown to work in other *Nicotiana* species, potato, green pea, *Arabidopsis* and lettuce (Xu et al. 2012). With the cr-TMV / TVCV-based MagnICON™ system (Icon Genetics, Halle) it is possible to produce 5 mg GFP per gramm fresh leaf in *N. benthamiana* in 14 days (Marillonnet et al. 2004). This short-term availability of heterologous proteins is advantageous when sudden demand appears. The need of vacuum infiltration equipment combined with a mandatory processing makes this approach more cost intensive than the stable nuclear expression.

The host defines total biomass yield, storage properties, cultivation costs, length of production cycle, cost of downstream processing and edibility (Obembe et al. 2011; Benchabane et al. 2008).

Tobacco has a high soluble protein level, a high biomass yield of up to 100 tons of leaf biomass per hectare and as a model organism a variety of well-established transformation protocols (Tremblay et al. 2010). Due to the natural toxic alkaloids in the leaves, tobacco is not considered as food plant and therefore can not be able to breed with food-chain plants (Obembe et al. 2011). Tobacco is a good example, for the host defines the drug delivery way. The expression of pharmaceuticals in tobacco leaves normally makes purification necessary and only allows an administration via injection. An oral application of tobacco leaf material seems possible by using genetically modified tobacco variants (Menassa et al. 2001), with a very low nicotine concentration like cultivar MD609, which contains almost no nicotine (Kim et al. 2010). Due to the low level of toxic compounds, accumulation of the therapeutics in the seeds is another way to enable oral delivery with tobacco (Rossi et al. 2013).

Seeds such as those of legumes are known to store much protein (up to 40 % of their dry weight (Lau and Sun 2009) and are highly suitable for the recombinant production of proteins (Mikschofsky and Broer 2012). One of them is pea, wich is independent from nitrogen fertilization and also has a high protein yield of approx. 25 %

(www.grainscanada.gc.ca). Because of the capacity to store high amounts of recombinant proteins and a high seed yield per hectar, the pea seed is suitable to produce pharmaceuticals (Mikschofsky and Broer 2012; Stöger et al. 2005). Pea is a leguminous plant and therefore acts as a self-fertilizer. An outcrossing of transgenes, under field conditions was not observed (Polowick et al. 2002). To increase the level of containment and yield, Mikschofsky and Broer 2012 recommend the cultivation of plants in the greenhouse chambers.

Plant suspension culture is an alternative system of recombinant protein production compared to transient expression and stable nuclear transformation. Carrot cells are a capable platform for biopharmaceuticals, because of the relatively simple callus initiation and maintenance using stem segments, cotyledons, or roots as explants, as well as the high regeneration potential by either organogenesis or embryogenesis. These properties made carrot a model species suitable to study a number of processes such as morphogenesis, somatic embryogenesis, somaclonal variation, and protoplast recovery (Rosales-Mendoza and Tello-Olea 2015). There is the opportunity to establish a suspension culture, which offers full containment of the transgene and a high rate of cell growth combined with product uniformity (Shaaltiel et al. 2007). Protalix approved the first plant-based pharmaceutical, the glucocerebrosidase (GCD) which is lacking in patients with Gaucher´s disease. Due to non-existence of toxic compounds an oral vaccination is possible.

Yield improvements

Yield (mg/kg biomass) is a critical point for the success of a vaccine and will be determined by the host, the expression organ, the used promoter and the expression/transformation strategy as mentioned in the chapter before. But also expression itself and protein stability are factors influencing the yield (Schillberg et al. 2005; Faye et al. 2005a).

The expression of a heterologous protein is regulated at transcriptional, translational and post-translational level (e.g. glycosylation) (Desai et al. 2010). The transcriptional level includes copy number and integration locus, initiation of transcription, RNA processing and RNA stability (Desai et al. 2010). Optimal transgene copy number and integration locus is difficult to control and could achieved by selecting best performing lines from a population of transgenic plants. But with an appropriate design of the expression cassette the promoter activity, RNA processing (e.g. integration of intron-mediated enhancements), RNA stability (e.g. polyadenylation sites), translational efficiency (e.g. codon usage can influence the transcriptional and translational level and influence the expression rate (Desai et al. 2010; Schillberg et al. 2003; Stöger et al. 2002).

Protein stability is mainly influenced by numerous proteolytic processes inside the plant cell (Benchabane et al. 2008). Localizing the transcript to a cell organelle, compartment or tissue could lower the risk of degradation, because of lower abundance of residing proteases or lower activity due to desiccant conditions (Xu et al. 2012; Benchabane et al. 2008; Petruccelli et al. 2006; Ma et al. 2003). Co-expression of protease inhibitors, chaperons or fusion partners could enhance the protein stability (Benchabane et al. 2008; Mishra et al. 2006). A rapid expression via transient expression systems (e.g. MagnICON) could also shorten the time of contact between a heterologous protein and host proteases (Nausch et al. 2012a).

For molecular farming in plants a high accumulation of the pharmaceutical is necessary. Accumulation of recombinant proteins in plants often results in much lower accumulation levels than 1 % of TSP (Daniell et al. 2001), what often is a reason for not realizing a market launch (Desai et al. 2010). The optimal production platform has to be determined experimentally and subsequent yield improvements are often necessary.

Plant-based ZP spaying

The idea of using plants to express ZP vaccines for spaying animals is a traditional approach. Fitchen et al. 1995 expressed virus-like particles (VLP) containing a contraceptive murine ZP3 peptide (aa 331-343) (Millar et al. 1989). This protein was robust and accumulated in high levels as a rod-like particle in tobacco. Parenteral immunization of BALB/c mice exhibited a relatively low level of anti-ZP3 antibodies and exhibited no significant effect on mice fecundity. The reason for using plants as production platform are the high scalability and cheap production, which provide a virtually universal access of bulk protein (Fitchen et al. 1995). Furthermore they offer oral delivery, when ZP vaccine is accumulated in edible plant parts (Polkinghorne et al. 2005; Smith et al. 1997). Transgenic tomato accumulated 37.8 $\mu g/g_{DW}$ of a heat-labile enterotoxin B subunit (LTB) / murine ZP3 fusion protein of (Walmsley et al. 2003) and achieved a reduction of 45 % of mice litter size after oral immunization (Patent application WO2002083072A2, example 20).

If a mammalian-like glycosylation pattern for ZP vaccines is essential or at least supportive, plants could offer this feature. But the influence of glycosylation at the ZP glycoproteins is contentious, because the literature shows different results. A glycosylation of the native ZP3 gained from pig ovaries (Paterson and Aitken 1990) or expressed in a baculovirus expression system (Prasad et al. 1995) leads to a higher and enzymatically or chemically de-glycosylated ones to a lower immunogenicity (Kerr 1999; Keenan 1991; Bhatnagar et al. 1991; Yurewicz et al. 1987; Sacco et al. 1986). Recombinant expression of rabbit ZP1 (VandeVoort et al. 1995; Schwoebel 1992), bonnet monkey ZP3 (Kaul et al. 1997) and ZP1 (Gupta 1997) in a bacterial expression with no glycosylation is only poor immunogenic (Paterson et al. 1998). However, some *E. coli* produced vaccines had strong contraceptive effects (see above: “Recombinant ZP vaccines”). According to Hasegawa et al. 2002 the immunocontraceptive efficacy of the ZP antibody raised against the ZP vaccine mainly depends on high similarity to the target on the ZP, so a mammalian-like glycosylation could be important for spaying, when the target ZP epitope is also natively glycosylated (Hardy et al. 2003).

The creation of spaying vaccines in food-chain plants or edible plant organs makes a closed production environment necessary. The breeding in greenhouses complies this requirement (Ma et al. 2003), although the scalability is more limited than open field cultivation. A second approach is the production in a cell culture, like carrot cell suspension, which also offers full biocontainment of the transgene (Rosales-Mendoza and Tello-Olea 2015).

However, every new transgenic approach needs a case specific study. For a spaying vaccine, both questions of species-specificity and risk assessment of transgenic outbreak (dependent of host and cultivation system) into other edible plants have to rule out.

Aims

The main aim of this study is to choose an antigen for spaying different animal species and to design a potently immunogenic vaccine in plants. The novel designed vaccine will be expressed in different stable and transient plant expression systems to find the optimal production platform. Based on the assumption, vaccine efficacy is independent from production system, the vaccine will be produced by the system with the highest production rate, comprising of yield per biomass and production time, to test this in a rodent model. In these animal trials antibody titer development and effect on fecundity will be determined. Due to the determined results from production (accumulation level) and efficacy (spaying) site an economic evaluation will be possible.

The aims of the current study are as follows:

1. Designing a ZP vaccine in fusion with adjuvants.
    a. Selecting optimal ZP antigens with high potential of spaying.
    b. Designing a vaccine, which is expressible in plants and will elicit immune response, with the possibility of oral vaccination
2. Transient and stable expression in different transgenic plant systems
    a. Cloning transformation vectors
    b. Agrobacterium-mediated plant cell transformation
3. Detection of ZP fusion protein / ZP vaccine
    a. Protein isolation
    b. Quantitative determination of accumulated ZP vaccine via ELISA
    c. Qualitative assessment of ZP vaccine via Western blot analysis
4. Purification of ZP vaccine proteins
    a. Small scale purification via affinity chromatography
    b. Select vaccine production platform by yield/time and vaccine concentration/biomass
    c. Upscaling with results from vaccine characterization (point 5)
5. Biochemical characterization of ZP vaccine
    a. Vaccine stability *in vivo* & *in vitro*
6. Evaluation of efficacy
    a. Testing potential to elicit immune response
    b. Testing the immunocontraceptive effect

3. Material & Methods

3.1 Vector construction and cloning

DNA isolation

To isolate plasmid DNA from *E. coli*, NucleoSPin® Plasmid EasyPure (Macherey-Nagel) protocol was used. DNA mass was measured by Nanodrop at 260 nm.

Plant DNA was isolated (Murray and Thompson 1980) using the Khanuja buffer (Khanuja et al. 1999). Leaf sample (2 cm^2) was homogenized in a bead mill (Precellyse, Bertin Instru.) and 300 µl Khanuja buffer running 2 x 30 sec with 6,500 rpm. Homogenate was incubated for 30 min at 65 °C. 300 µl Chlorophorm: Isoamylalcohol (24:1) were added in the samples and overhead rotated for 1 min. Samples were centrifuged for 5 min at 13,000 rpm at 4 °C. Aqueous phase was transferred to a new Eppendorf tube and gently mixed with 1 volume of isopropyl alcohol. DNA was precipitated for 30 min in a freezer and pelleted by spinning at 13,000 rpm for 15 min at 4 °C. Supernatant was discarded and pellet was washed with 200 µl 70 % ice cooled ethanol. Pellet was air dried and resuspended in 30 – 50 µl ddH_2O.

Transformation of bacteria

E. coli TG1 and BL21 were transformed via heat shock method (Hanahan 1983). Competent cells were thawned 30 min on ice. Up to 10 µl Plasmid-DNA solution was added. Heat shock was 2 min on 42 °C with subsequent cooling for 5 min on ice. After addition of 1 ml LB Medium, cells were incubated at 37 °C for 1 h on a shaker. Postively transformed cells were selected on LB plates containing antibiotics.

A. tumefaciens ICF320, LBA 4404, EHA 105 were transformed via freeze shock method. Bacteria were grown over night in LB media. After addition of 10 µl DNA to 500 µl bacteria suspension, mixture was kept on ice for 5 min. Subsequently it was frozen for 5 min in liquid nitrogen and thawn for 5 min at 37 °C on a shaker after adding 1 ml LB media. Cells were grown for 4 h at RT. Positively transformed bacteria were selected on agarose plates containing antibiotics.

Colony-PCR

To identify positive *E. coli* clones, each single colonies were picked with a wooden, steril toothpick and boiled for 5 min in ddH_2O. 1 µl was used in a PCR as template. For testing *A. tumefaciens* clones were boiled in 50 µl lysis buffer (0.05 M NaOH + 0.25 % SDS), cooled on ice. After addition of 450 µl ddH_2O, cell debris was pelleted by centrifugation. 1 µl of supernatent was used as template in a colony PCR (Table 7).

DNA digestion and ligation

To identify a plasmid by characteristic fragments after enzymatic digestion, 1 – 5 µg DNA was used with 0.5 µl enzyme. Batch was incubated for 1.5 h at 37 °C. Restriction pattern was checked in an agarose gel electrophoresis.

For a subsequent batch ligation, the amount [fmol] of vector and insert was determined with the aid of nanodrop (Thermo Fisher™). The ratio between vector and insert was 1:1 till 1:5. Approach was incubated for 1.5 h at 37 °C and enzymatic reaction was stopped by 10 min at 65 °C or higher, dependent of enzyme. Ligation was performed with T4 ligase

(Thermo Fisher™) overnight in the fridge. T4 Ligase was deactivated by 65 °C incubation for 10 min.

When linearized vector and insert have to be purified by gel electrophoresis, QIAquick PCR Purification Kit was used to extract the bands from the agarose gel.

VacZP2

The fusion gene was synthesized by Eurofins (Ebersberg, Germany) and delivered in a pUC57 vector. The fusion gene *vacZP2* (Figure 4D) was inserted into binary transformation vectors pSingle35S (Figure 4A) and pARC (Figure 4B) by *Xma*I and *Sma*I restriction sites (Figure 6A, B). To obtain seed specific expression the vector pARC was cloned by Antje Höhne (unpublished data), a pLH9000 (Hausmann and Töpfer 1999) derivate, carrying a seed specific *arcelin5*-I promoter from *Phaseolus vulgaris* (Jaeger et al. 2002; accession #AF458478). For transient expression we cloned the fusion gene via *Bsa*I sites Engler et al. 2008; Figure 6) in the cr-TMV / TVCV-based MagnICON™ vector pICH29912 (Figure 4C), which was kindly provided by Nomad Bioscience (Halle/Saale, Germany).

$_{\Delta}$ZP2$_{only}$P

The coding region of $_{\Delta}$pZP2 was amplified via recombinant PCR from a pET28a + *vacZP2* vector with the primer pZP2_pICH31120f and pZP2_pICH31120r (Table 9). The PCR product was cloned into a pJET1.2 vector (CloneJET PCR Cloning Kit, Thermo Fisher; Figure 12). The coding region of $_{\Delta}$pZP2 was combined with SEKDEL and histidine tag (Figure 4E) by *Bsa*I restriction into pICH31120 (Figure 4F), a transient expression vector (Nomad Bioscience, Halle), and transformed to *Agrobacterium tumefaciens* strain ICF320.

VacZP3-P

To amplificate *vacZP3-P* PCR program 2 was conducted (Table 6). PCR product *vacZP3-P* (Figure 14) was cloned blunt end with CloneJet-Kit (Thermo Fischer™) into pJET1.2.

VacZP3-B

For expression in *E. coli* we deleted the ER-targeting signal by PCR with primer ÜEx_synZP3_NdeI and ÜEx_synZP3_XhoI (Table 9) and integrated the product in vector pJET1.2 by blunt end cloning (Figure 20). *VacZP3-B* was cloned into pET28a by *Nde*I and *Xho*I restriction sites and selected by kanamycin. *VacZP3-B* was integrated into the transformation vector pET22b by *Nde*I and *Xho*I restriction sites and transformed into *E. coli* BL21. Clones were selected by ampicillin. Transformation vector is an isopropyl β-D-1-thiogalactopyranoside (IPTG) -inducible overexpression vector (Novagen). The integrity was validated by sequencing (Eurofins MWG, Ebersberg, Germany).

3.2 Stable transformation & cultivation

Carrot cell

For the experiments a callus culture of petioles of *Daucus carrota* (var. Rotin) made by Jafargholi Imani (University Giessen) was used. Suspension cell culture was established as described by Mikschofsky et al. 2009c with B5 media (Gamborg et al. 1968) instead of Litvay's medium. Suspension cell transformation was performed with *Agrobacterium tumefaciens* strain LBA4404 described by Mikschofsky et al. 2009c. After transformation a homogeneous and friable callus material light, yellow in color, was placed in a thin layer

on solid Gamborg B5 with 0.47% (w/v) Gamborg B5 medium (incl. vitamins), 2% (w/v) sucrose, 0.6% (w/v) agarose, 2.3 µM 2.4-D, 50 µg mL^{-1} kanamycin and 250 µg mL^{-1} cefotaxime for selection of transformed callus cells. After a growth period of approximatly four weeks in growing chambers at 24/18°C under dark conditions, small single transgenic callus clusters where transferred onto a new plate.

Tobacco

The vectors p35S-VacZP2 (Figure 4A), pARC-VacZP2 (Figure 4B) and pARC-VacZP3-P (Figure 5A) were introduced into tobacco *Nicotiana tabacum* cv. Petit Havana SR1 (Maliga et al. 1973) by the *Agrobacterium tumefaciens* strain LBA4404 conducted as previously described (Wohlleben et al. 1988). Regenerated plants were set on soil and cultivated in greenhouse until they mature.

Pea

Transformation of *Pisum sativum* cv. Greenfeast with pARC-VacZP3-P (Figure 5A) and cultivation of pea was conducted as described in Mikschofsky et al. 2009b initially described by Polowick et al. 2000. Minor adaptations of the Mikschofsky protocol were carried out. Cultivation media P1, P2, P3 (alias B5/2T) contained no *L*-phosphinothricin (L-PPT). Shoot elongation medium (MS7T, Polowick et al. 2000) was not used. Agarose concentration in P3 medium was reduced to 1.5 % (w/v).

E. coli

After transforming pET-VacZP3-B (Figure 5D) to *E. coli* BL21 bacteria were cultivated and IPTG-induced like previously described by Nausch and Broer 2016b. Pellet was stored at -80 °C for further processing.

3.3 Transient tobacco transformation

Seven to nine weeks old *N. benthamiana* plants transfected with pICH-VacZP2 (Figure 4C), pICH-$_{\Delta}$ZP2$_{only}$P (Figure 4F) and pICH-VacZP3-P (Figure 5C) according to Ponndorf et al. 2016 with minor adaptations, respectively. In our method the atmosphere under the cabin´s hood after evacuation was gradually restored within 5 minutes.

Leaves were snap-frozen with liquid nitrogen directly after harvest and stored at -80 °C. Frozen leaves were freeze-dried for three till five days. Isogenic leaves (same day of harvest) were pooled and pulverized using a blender. Material was stored at room temperature (RT) in the dark.

3.4 Protein isolation

Carrot calli

200 mg calli was homogenized in a bead mill (3 x 30 sec, 6300 rpm, 5 min cooling pause between each round) with 250 µl 1x PBS (10 mM Na_2HPO_4, pH 7.4, 2 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl), sea sand and zirconia beads. Protein extract was obtained by spinning 3 times for 5 min at 15000 xg and 4 °C.

Tobacco

Fresh leaf material was ground by an Eppendorf tube pestle cooled in liquid nitrogen. Freeze-dried plant material was pulverized by a blender. Tobacco seed material was

homogenized in cooled protein extraction buffer by a ceramic bead mill (Precellyse, VWR, Darmstadt, Germany). After addition of 500 µl protein extraction buffer (50 mM NaH_2PO_4, 250 mM sucrose, 300 mM NaCl, 5 mM Imidazol) to i) 100 – 150 mg fresh material ii) 23 – 30 mg freeze-dried or iii) 50 mg tobacco seeds, samples were mixed for at least half a minute and subsequently centrifuged (5 min, 15000 xg) 3-times at 4 °C. Total soluble protein (TSP) content of supernatant was determined by Bradford 1976 method using Coomassie and bovine serum albumin as standard.

Pea

Sample material from dry pea seeds was obtained by milling cotyledons with a mini-drill (Dremel, US-WI) (Figure 3). Care was taken to not damage the embryo. Resulting milling dust was collected in 1.5 ml Eppendorf tubes and cold protein extraction buffer was added. Sample was vigorously shaken for 10 min and 3 times centrifuged (5min, 15,000 xg) at 4 °C. TSP in supernatant was determined by (Bradford 1976) method using Coomassie and bovine serum albumin as standard.

Figure 3: Milled sample from pea cotyledons using a mini-drill.

3.5 Ni-NTA purification

Plant

Freeze-dried leaf material was mixed with protein extraction buffer by vortexing or using a blender (Polytron Pt-MR 2100, Kinematica AG, Switzerland). The supernatant was loaded via a Perista Mini Pump SJ-1215 (Burkhard Instrumente AG; Zürich / Bional AG) at 2.5 ml min^{-1} on a column (Biorad™, Prod# 727-1517) containing 5 ml of pre-equilibrated Ni-NTA resin (Probond™ resin, Prod# 46-0019, Life Technologies). The resin was washed with 100 ml of wash buffer (50 mM NaH_2PO_4, 10 mM imidazol, 300 mM NaCl, pH 8) at 2.5 ml min^{-1}. To wash the bound protein from the column 20 ml elution buffer (50 mM NaH_2PO_4, 300 mM imidazol, 300 mM NaCl, pH 8) was added with a reduced flow rate of 1 ml min^{-1}. The elution fraction was concentrated and desalted in a Vivaspin 20 (GE healthcare, Freiburg, Germany, membrane cutoff: 10 kDa, Prod# 28932360) ultrafiltration falcon by spinning at 4600 rpm in a swing-out centrifuge (Heraeus multifuge 3 L-R, Hanau, Germany). The sample was concentrated to a volume of 2 -5 ml and refilled to 20 ml with cooled PBS buffer. This procedure was done two times at 4 °C.

E. coli

Protocol for purification of TT-KK-ZP3 was provided by Vidisha Minhas (PhD student of S. K. Gupta; Dehli). After resuspending the pellet in binding buffer (20 mM NaH_2PO_4, 500 mM NaCl, 20 mM Imidazole, 8 M urea, pH 8), cells were lysed by sonication (8 x 30 sec; 40 W). Cell suspension was rotated for 2 hours by an over-head rotator. After spinning (10,000 xg; 20 min; 4 °C) supernatant was collected and incubated on an over-head rotator with Ni-NTA resin for 3 h. Mixture was loaded on an equilibrated BioRad® Poly-

Prep column. Column was washed twice with 4 ml washing buffer (20 mM NaH_2PO_4, 500 mM NaCl, 40 mM Imidazole, 8 M urea, pH 6.3). Protein was eluted 4 times with 0.5 ml of elution buffer (20 mM NaH_2PO_4, 500 mM NaCl, 250 mM Imidazole, 8 M urea, pH 4.5). Fraction with highest heterologous protein was dialyzed against renaturation buffer (20 mM PBS, 0.1 mM glutathione (GSH), 0.01 mM glutathione disulfide (GSSG), 1 mM EDTA, 10 % sucrose, 4 M urea, pH 8) for 2 h. Buffer was refreshed with gradual reduction of urea concentration (2, 1, 0.5 M). Final buffer was 20 mM PBS burfer (20 mM NaH_2PO_4, 2 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl). Sample was stored at -20 °C until use.

For purification of VacZP3-B bacterial pellet was resuspended in lysis buffer (50 mM Tris-KCl, 300 mM NaCl, 10 mM Imidazole, pH 8) and cells were destroyed by 3-times sonication (Bandelin Sonotrode HD2200 + micro tip MS73) for 30 sec at 10 % power. Between the interval tip was cooled down. Cell debris was separated by 15 min at 4,600 xg and 1 h at 16,000 xg subsequent spinning. Supernatant was loaded on an equilibrated Ni-NTA column and washed with 10 resin-volumes of buffer I (like lysis buffer but 20 mM Imidazole) and buffer II (like buffer I but 75 mM imidazole). VacZP3-B was eluted with 2 resin-volumes of elution buffer (like lysis buffer but 300 mM imidazole). Buffer was exchange by ultrafiltration (Vivaspin 20, 30 kDa cut-off, Satorius) to 1x PBS (pH 7.2).

3.6 Enzyme-linked immunosorbent assay (ELISA)

Anti-CTB & GM1

Content of CTB and assembling to pentamers was determined by anti-CTB ELISA and GM1-ELISA (Mikschofsky et al. 2009b).

Anti-VacZP2 & anti-$_{\Delta}$ZP2$_{only}$P

Serum IgG against VacZP2 was determined by coating VacZP2 and $_{\Delta}$pZP2$_{only}$P to microtiter plates (detailed information in Supplement). Secondary Antibodies were anti-Mouse-IgG conjugated to horseradish peroxidase (HPR).

Anti-VacZP3-P & anti-TT-KK-ZP3

This ELISA was used to detect anti-VacZP3-P antibodies in blood serum. VacZP3-P was diluted in 1x PBS to 100 ng/ml and loaded to 96-well microtiter plate 100 µl each well. After incubation for 2 h at 37 °C wells were washed 3-times with 200 µl PBS + 0.5 % Tween20. Blocking step was conducted with 300 µl 1 % BSA in 1x PBS for 45 min at room temperature (RT) on a shaker. After washing step animal serum diluted to i) 1:6,000 in KVP (Konjugatverdünnungspuffer, DRG Instruments GmbH, Marburg) for anti-VacZP3-P ELISA and ii) to 1:500 in KVP for anti-TT-KK-ZP3 ELISA, respectively. Diluted sera were loaded and incubated for 1.5 h at RT on a shaker. After washing step anti-mouse-POD antibody (1:2000 in KVP) was incubated for 45 min at RT on a shaker. After washing step 100 µl 3,3′,5,5′-Tetramethylbenzidine (TMB) was loaded for 3 min and stopped with 250 mM H_2SO_4. Detection was done at 450 nm.

3.7 Western blot analysis

Protein extracts were separated in an 12 % SDS-PAGE (Ponndorf et al. 2017) with the adaptation of 10 minutes of heat denaturation and transferring the proteins by tank blotting (BioRad, Hercules, USA) (100 V, 40 min) to the membrane. To detect the *N*-terminal CTB domain the membrane was blocked with 4 % skim milk (w/v) in TBST (20

mM Tris, 137 mM NaCl, 0.05% Tween20, pH 7.6) for 1.5 h at room temperature (RT). Primary anti-CTB antibody (Fizgerald, 20-CR18) was diluted 1:2,000 in buffer II (2 % skim milk (w/v) in TBST) and incubated for 2 h. After washing with buffer II, secondary antibody anti-goat-IgG, diluted 1:5,000 in buffer II was incubated for 1 h. Membrane was washed with TBS (20 mM Tris, 137 mM NaCl, pH 7.6) and ECL was done. Anti-histidine and concanavalin A Western blot analysis was conducted as (Nausch and Broer 2016b) with minor adaptations, including membrane for anti-histidine detection was blocked with 3 % skim milk (w/v) in TBST.

3.8 Degradation assay

VacZP2 was extracted with optional addition of protease inhibitor (cOmplete, Roche, 4693124001). The TSP extract was incubated at RT and 4 °C, respectively. Incubation was stopped by addition of SDS containing sample buffer and boiled for 10 min. Samples were loaded on 12 % SDS-PAGE and blotted on a nitrocellulose membrane. Subsequently, an anti-CTB Western blot was conducted.

3.9 Densitometric CTB determination

CTB was detected using an anti-CTB Western blot with gradual dilutions of a CTB standard (Sigma-Aldrich) and VacZP2 after purification. The film with medium signal strength was captured by a digital camera (Nikon E4500) in front of a white paper. The distance of the film and the paper was 20 cm to allow transillumination. The density of the CTB standards (50, 100, 300 ng) was compared to the density of the CTB monomer signal in the vaccine with ImageJ (Abràmoff et al. 2004). The estimated mass of CTB was related to the total loaded mass of CTB within the VacZP2 lane, which was previously determined in the anti-CTB ELISA.

3.10 Northern blot analysis

RNA isolation and Northern blot analysis was conducted described by Ponndorf et al. 2016. Dig-labeled CTB specific probe was amplified by Primer CTB_new_fw and CTB_new_rev (Table 9).

3.11 Animal trial

VacZP2

Four test groups of female FvB/J mice were primed subcutaneous with 264 µg (140 µl) purified VacZP2 in 1x PBS (pH 7.2) or the equal volume 1x PBS with or without 10 % Polygen (MVP Lab). All mice were boostered intraperitoneal with the same dose after 26 and 51 days. During this immunization schedule the animals were retrobulbary bleeded on day 0, 21, 37 and 65 (Figure 30C).

Two groups of 8 female golden hamsters were primed with 20 µg (200 µl) VacZP2 and the same volume of 1x PBS, respectively. All doses included 10 % Polygen (MVP Lab). After 21, 35, 49, 63 days animals were boostered with the same doses. The animals were bleed on day 0, 21, 35, 49, 63 and 84 (Figure 31C). One animal per group were euthanized and ovariectomized on day 21 and 49. After 42 days 4 and 5 females immunized with and without VacZP2 were mated with male hamsters with proven fertility, respectively. On day 84 two VacZP2 immunized animals and 1 animal from the PBS group were euthanized and ovariectomized. The ovaries were fixated in 4 % formaldehyde and embedded in

paraffin wax. Tissue sections (5 µm) were stained with hämatoxylin/eosin using standard protocols.

VacZP3-P

Three groups of ten female BALB/c mice were subcutaneously primed with two, seven and 21 µg plant-made VacZP3-P, respectively. One group was vaccinated with 25 µg TT-KK-ZP3, a bacterial vaccine with proven sterilizing efficacy (Gupta et al. 2013), to verify responsiveness of the BALB/c mouse strain. One group received 1x PBS (pH 7.2). All doses included 10 % Polygen (v/v) (MVP Lab). At day 43 and 63 all animals were subcutaneously boostered with the same doses received for priming. Animals were retrobulbary bled once before and three times after each treatment at day 0, 41, 58 and 72. After 82 days females were mated with males of proven fertility. For an overview see Figure 37C.

3.12 Statistics

To identify a significant difference between tested groups ($p \leq 0.05$), data were analyzed either in an univariant ANOVA (including post-hoc Tukey-HSD) or with a non-parametric test (Mann-Whitney-*U*) with SPSS (IBM, Version 22).

4. Results

4.1 Gene design and expression strategy

We designed two fusion vaccines, named VacZP2 (Figure 4D) and VacZP3 (Figure 5B), which hold the coding region for a truncated porcine ZP protein 2 ($_{\Delta}$pZP2, Ile_{36} – Ala_{168}, P42099, uniprot.org) and full-length, porcine ZP3 (Gln_{23} – Ser_{332}; uniprot.org P42098) as antigens. Different studies with porcine ZP2 (Miller and Killian 2002; Hasegawa et al. 2002; Hasegawa et al. 2000; Shigeta et al. 2000; Hasegawa et al. 1995; Koyama et al. 1991) and other animal's ZP2 like mouse (Lea et al. 2002; East and Dean 1984), bonnet monkey (Govind et al. 2002), brushtail possum (Duckworth et al. 2007), rabbit (Mackenzie et al. 2006) and human (Chakravarty et al. 2005; Tsubamoto et al. 1999) have shown that this component of the ZP is a potential target to decrease mammalian fertility. At the *N*-terminus of the homologous murine ZP2 the taxon-specific sperm recognition is located (Avella et al. 2014). This was also confirmed by incubation of humane oocytes with anti-porcine $_{\Delta}$ZP2 (amino acid 1 - 198), which did not affect sperm binding (Hasegawa et al. 2000). ZP3 plays also a crucial role in sperm binding (Chiu et al. 2014; Chen et al. 1998; Kinloch et al. 1995) and matrix structure (Liu et al. 1996; Rankin et al. 1996).

Immunocontraception studies on Kangaroo (Kitchener et al. 2009a), bonnet monkey (Afzalpurkar et al. 1995; Bagavant et al. 1994), squirrel monkey (Sacco 1987), marmoset (Paterson et al. 1992), dog (Shrestha et al. 2015; Srivastava 2002) and mouse (Hardy et al. 2003; Millar et al. 1989) with ZP3 as target antigen show high spaying potential. Recombinant porcine ZP3 sterilized mice (Gupta et al. 2013; Clydesdale et al. 2004), but failed to operate in horses (Joone et al. 2017). Dr. Kirkpatrick (History of PZP, unpublished) does not recommend an extrapolation of immunogenicity of a new ZP vaccine from other studies, because of a variety of factors influencing an effective immunocontraception. Factors are glycosylation and conformation determined by the production source of the antigen (e.g. native, recombinant), an allo- or heteroimmuniziation, immunization schedule and immunomodulating techniques (e.g. adjuvant, formulation) (Curtis et al. 2007; Hardy et al. 2003; Dunbar et al. 1994; Dunbar and Raynor 1980). So, every new ZP vaccine has to be individually evaluated, but our chosen porcine $_{\Delta}$pZP2 and ZP3 are undoubtedly important for fertilization.

To circumvent MHC restriction, thereby increase the number of individual responders and modulate the immune response (cellular vs. humoral), a sophisticated antigen design is necessary (Bagavant 1997). Amoung other things, this could be done by creating multiepitope antigens (Hardy et al. 2008; Hardy et al. 2004) or promiscuous T-cell epitopes coupled to the ZP epitope, like e.g. bovine RNase (Lou et al. 1995a; Chen et al. 1991), KLH (Millar et al. 1989) or diphtheria toxin (Srivastava 2002; Govind and Gupta 2000). *N*-terminally fused in frame to the antigens is the non-toxic subunit of cholera toxin (CTB) (P01556, uniprot.org) as a humoral adjuvant (Holmgren et al. 2005) enabling a mucosal delivery (reviewed in Baldauf et al. 2015) and *C*-terminally 45 bp of the tetanus toxoid (TT) (Gln_{830} – Leu_{844}, P04958, uniprot.org) as an adjuvant for an effective helper T-cell activation (Fraser et al. 2014). The translation is ER-targeted and the protein retains in the ER via the retention signal SEKDEL (Denecke et al. 1992). This is necessary for CTB pentamer assembling (Mikschofsky et al. 2009a) and ER-targeting is still favorable, because this compartment is able to accumulate high amounts of recombinant protein (Wandelt et al. 1992) and resident chaperones together with lower proteolytic activity

stabilizes proteins and antibodies (Benchabane et al. 2008), like in many studies (Nausch et al. 2012b; Mikschofsky et al. 2009a; Stöger et al. 2002; Conrad and Fiedler 1998; Schouten et al. 1996). To increase translational efficacy and protein stability additional sequence for Ala-Ser-Ser (Sawant et al. 2001) was integrated directly after start codon during gene design. To alleviate steric hindrance during auto-assembly of the CTB domains a glycine-proline-glycine-proline (GPGP) linker (Ljpscombe et al. 1991) was introduced between CTB and $_{\Delta}$pZP2 and ZP3, respectively. The omission of the GPGP linker between CTB and a HIV antigen resulted in a reduced ability of the CTB fusion protein to form a pentamer (Lee et al. 2014). The di-lysine linker was integrated to probably improve antigen processing of the Tetanus Toxoid and thereby stimulate the cellular immune response (Sarobe et al. 1993; Takahashi et al. 1988). With the protease site AcTEV (Nayak et al. 2003) a separation of TT is possible to allow an influence on the adaptive immune response.

The histidine tag enables an easy purification via affinity chromatography. The protease site Factor Xa (Jesty and Nemerson 1976) opens the opportunity to deposit the histidine tag. Both fusion gene sequences were optimized by Eurofins GENEius software to plant codon usage and to prevent bad motifs, like artificial splicing sites or premature polyadenylation signals. The subsequent *in silico* testing with the prediction server NetGene2 (Hebsgaard et al. 1996), showed no putative splice products during plant expression.

To obtain a constitutive expression of VacZP2 in all transformed plant cells the fusion gene was integrated into pSingle35S vector, designated as p35S-VacZP2 (Figure 4A). For seed-specific expression of VacZP2 and VacZP3-P we used a pLH9000 derivate with a seed specific arcelin5-I promoter pARC (Jaeger et al. 2002) cloned by Antje Höhne (unpublished data), named pARC-VacZP2 (Figure 4B) and pARC-VacZP3-P (Figure 5A). Transient expression of VacZP2 (Figure 4C) and VacZP3-P (Figure 5C) was realized by the cr-TMV / TVCV-based MagnICON™ vector pICH29912 provided by Nomad Bioscience (Halle/Saale, Germany).

For determination of antibodies after vaccination with VacZP2, which are exclusively directed to the antigen domain $_{\Delta}$pZP2, the domain $_{\Delta}$pZP2 was transiently expressed in *N. benthamiana* via pICH31120 vector (Figure 4F). The resulting protein $_{\Delta}$pZP2$_{only}$P (Figure 4E) was coated to ELISA plates. The pICH31120 vector is equal to pICH29912, but has an integrated ER-targeting sequence from the apple pectinase, a histidine tag and SEKDEL sequence.

VacZP3-B (Figure 5B), the bacterial expressed VacZP3-P, was designed to act as a positive control in bioanalytical assays, e.g. Western blot. In order to get a bacterial fusion protein similar to the plantal one, ER-targeting sequence of *vacZP3-P* was deleted and cloned into pET22b (Novagen) (Figure 5D).

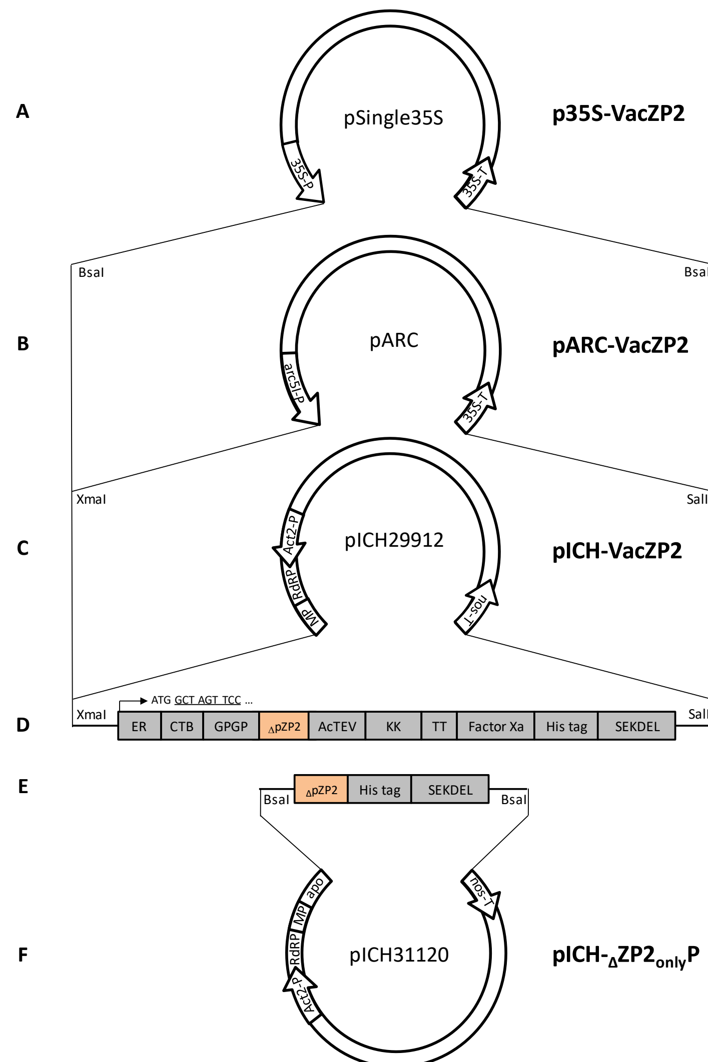


Figure 4: Vectors for VacZP2 expression. (A) p35S-VacZP2 for constitutive expression. 35S: 35S transcript of Cauliflower Mosaic Virus; P: promoter; T: terminator. (B) pARC-VacZP2 for seed specific expression. arc5-I-P: *arcelin5*-I promoter of *Phaseolus vulgaris*. (C) pICH-VacZP2 for transient expression. Act2: *Arabidopsis* actin 2; RdRP: RNA dependent RNA polymerase; MP: movement protein of turnip vein clearing virus (TVCV); nos: nopaline synthase. (D) Coding region of the fusion gene *vacZP2*. Underlined sequence: addition of the amino acids Ala-Ser-Ser; ER: CTB targeting sequence for ER secretion; CTB: cholera toxin subunit B from *Vibrio cholera;* GPGP: Glycine-Proline-Glycine-Proline hinge; $_{\Delta}$pZP2: N-terminal porcine Zona pellucida 2 coding region (36 – 168 aa); AcTEV: recognition site for a TMV AcTEV protease; KK: di-lysine linker; TT: tetanus toxoid (830 – 844 aa); Factor Xa: recognition site for a bovine protease; His tag: 6 Histidine residues; SEKDEL: ER retrieval signal. (E) Coding region for *$_{\Delta}$pZP2$_{only}$P*. $_{\Delta}$pZP2: N-terminal porcine Zona pellucida 2 coding region (36 – 168 aa); His tag: 6 Histidine residues; SEKDEL: ER retrieval signal. (F) pICH-$_{\Delta}$pZP2$_{only}$P for transient expression. apo: apoplastidal targeting sequence from apple pectinase.

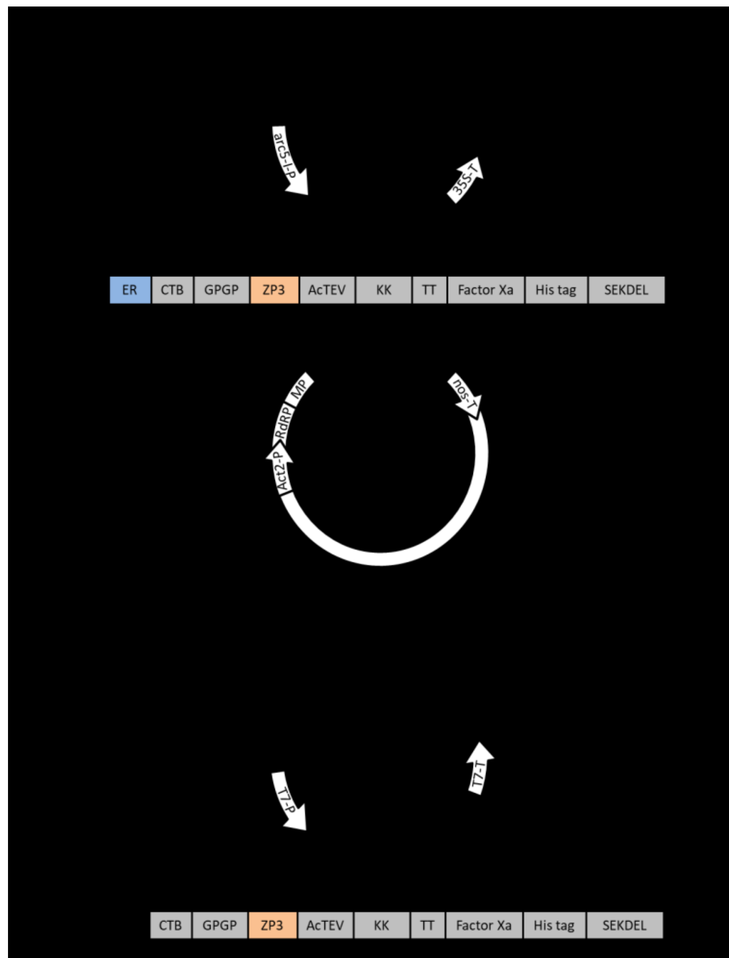


Figure 5: Vectors for VacZP3 expression. (A) pARC-VacZP3-P for seed specific expression. arc5-I-P: *arcelin5*-I promoter of *Phaseolus vulgaris*. (B) Fusion gene *vacZP3-P* for plant expression. Underlined sequence: addition of the amino acids Ala-Ser-Ser; ER: CTB intrinsic targeting sequence for ER targeting. CTB: cholera toxin subunit B from Vibrio cholera; GPGP: Glycine-Proline-Glycine-Proline hinge; ZP3: porcine Zona pellucida 3 coding region (23 – 332 aa); AcTEV: recognition site for a TMV AcTEV protease (Nayak et al. 2003); KK: di-lysine Linker; TT: tetanus toxoid fragment (830 – 844 aa); Factor Xa: recognition site for bovine protease (Jesty and Nemerson 1976); His tag: 6 Histidine residues; SEKDEL: ER retrieval signal; Act2-P: *Arabidopsis* actin 2 promoter; RdRP: RNA dependent RNA polymerase; MP: movement protein of turnip vein clearing virus (TVCV) ; NTR: untranslated region of TMV; nos: nopaline synthase; RB: right border; LB: left border; 35S: 35S transcript of Cauliflower Mosaic Virus, arc5-I: *arcelin5-I* gene from Phaseolus vulgaris; T7: T-phage lac operon, P: promoter T: terminator.

4.2 Vector construction

VacZP2

Constitutive expression of VacZP2 in carrot cells and *N. tabacum* plants was realized by transformation of p35S-VacZP2 (Figure 4A). The coding region was integrated into pSingle35S via *XmaI* and *Sal*I restriction sites and transformed to *E. coli* TG1. Streptomycin20/Spectinomycin50 (St20/Sp50) selected clones were verified by colony-PCR with primer pZP2_TT_fw + pZP2_TT_rev (Figure 7A). Clone 4 was used for further work. The plasmid was check by *Nde*I restriction to 531, 1760, 2117 and 6473 bp fragments. All fragments were detected (Figure 7B).

Figure 6: Overview of cloning strategies. (A) Constitutive expression of VacZP2. (B) Seed-specific expression of VacZP2. (C) Transient expression of VacZP2. p35S…cauliflower mosaic virus (CMV) promoter 35S; t35S…CMV terminator 35S; arc5-I…*arcelin 5*-I promoter; ParA, ParB, resolvase…repressor for transposon activity; Sm/Sp…streptomycin/spectinomycin resistance gene; km… neomycin phosphotransferase II (*NPT* II) for kanamycin resistance; bla…beta-lactamase gene for ampicillin resistance.

Plasmid from clone 4 was sequenced and transformed to *A. tumefaciens* strain LB4404. All clones growing on St100/Sp300 were PCR positive in a colony-PCR (Figure 7C). To ensure that tested agrobacteria were not contaminated with *E. coli* TG1 carrying p35S-VacZP2, a randomly amplified polymorphic (RADP)-PCR was conducted. No contamination of *E. coli* was detected in agrobacteria transformed with this vector and the empty vector control, respectively (Figure 8).

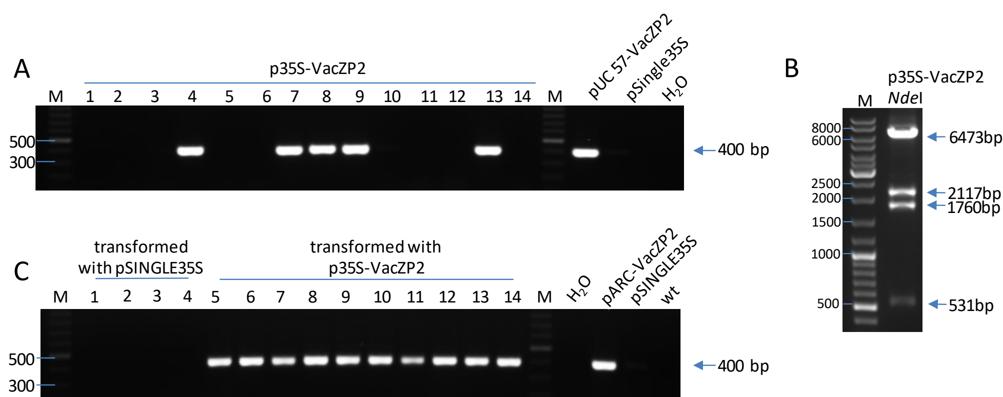


Figure 7: Verification of transforming *E. coli* with p35S-VacZP2. (A) Colony-PCR of p35S-VacZP2 transformed *E. coli* TG1 clones with primer pZP2_TT_fw + pZP2_TT_rev. Expected band is at 400 bp. 1-14…Clone 1-14. M…Marker DNA Ruler Ladder Mix. (B) Restriction of p35S-VacZP2 from clone 4 with *Nde*I. (C) Colony-PCR of p35S-VacZP2

transformed *A. tumefaciens* LB4404 with same primer like A. Blue arrows show expected product.

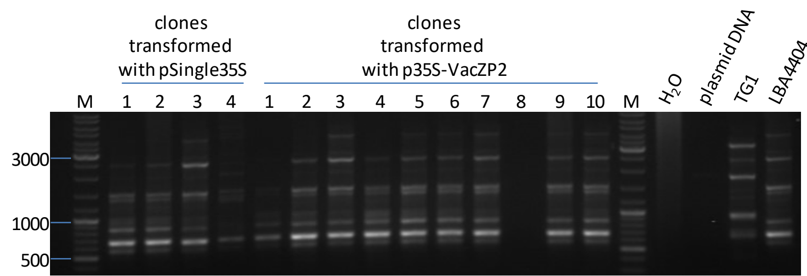


Figure 8: RAPD-PCR with daf9 primer on p35S-VacZP2 and pSingle35S transformed *A. tumefaciens* clones.

Seed-specific expression of VacZP2 was established by transformation of tobacco with pARC-VacZP2, a pLH9000 (Hausmann and Töpfer 1999) derivate, carries a seed specific *arcelin5*-I promoter from *Phaseolus vulgaris* (Jaeger et al. 2002) (accession #AF458478). *VacZP2* coding sequence was transferred via *Xma*I and *Sal*I restriction sites from pUC57-VacZP2 to pARC and selected on streptomycin/spectinomycin (Figure 6B). Positive clones were identified by colony-PCR amplifying the $_{\Delta}$pZP2 antigen with pZP2_TT_fw + pZP2_TT_rev primer pair (Figure 9A). Integration was also verified by *Nde*I restriction, where all expected fragments were detected (Figure 9B). Positive plasmid was sequenced and transformed to *A. tumefaciens* strain LBA 4404 for stable tobacco transformation.

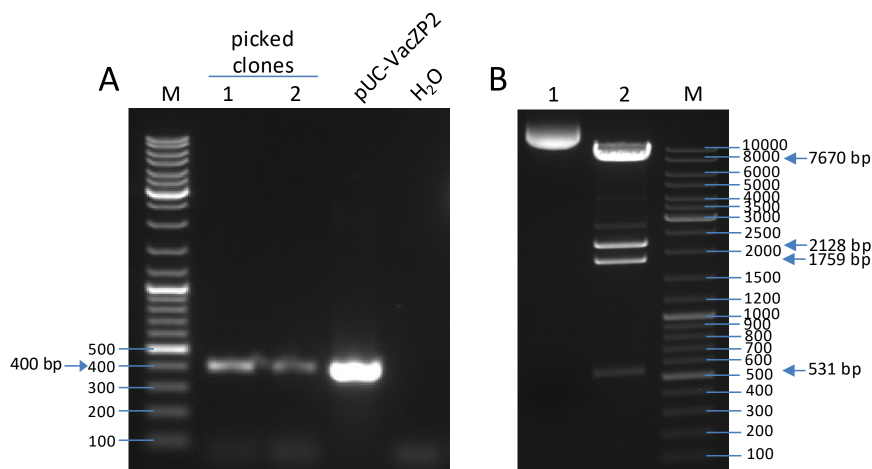


Figure 9: Verification of transforming *E. coli* with pARC-VacZP2. (A) Colony-PCR of two pARC-VacZP2 transformed *E. coli* TG1 clones with primer pZP2_TT_fw + pZP2_TT_rev. Expected band is at 400 bp. (B) 1...uncut pARC-VacZP2 plasmid DNA, 2...Restriktion of pARC-VacZP2 with *Nde*I. All expected bands of 7670, 2128, 1759 and 531 bp are detected.

Transient expression in *N. benthamiana* was realized with the transformation of pICH-VacZP2 (Figure 4C). VacZP2 was transferred from pUC57-VacZP2 to pICH29912 by *Bsa*I restriction earlier described by Engler et al. 2008. The ligation product was transformed to *E. coli* TG1 and selected on kanamycin. The positive colonies were identified by colony-PCR (Table 7) on the integration of *vacZP2* between the TMV

promoter and terminator with TMV_primer_fw + TMV_primer_rev (1571 bp) and the $_{\Delta}$pZP2 antigen by pZP2_TT_fw + pZP2_TT_rev (400 bp) (

Figure 10A). Plasmids of clone 2, 4, 5 and 6 were digested with *Eco*RI to double check the integration of *vacZP2*. A 689 bp fragment validates the plasmids from all tested clones (Figure 11). pICH-VacZP2 from clone 6 were transformed into A.t. ICF320 and selected on kanamycin and rifampicilin. Positive A.t. clones were identified like positive *E. coli* clones. A.t. clone 1 was chosen for plant transformation (Figure 10B).

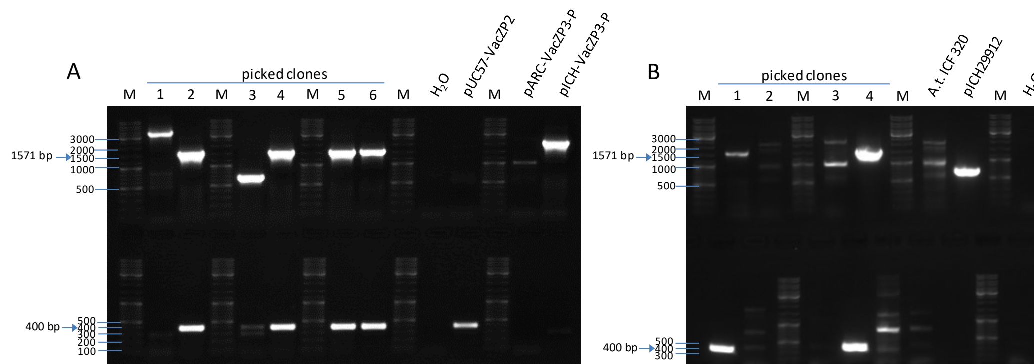


Figure 10: Colony-PCR of pICH-VacZP2 transformed (A) *E. coli* TG1 clones and (B) *A. tumefaciens* ICF320. Primer used: Upper panel…TMV_primer_fw + TMV_primer_rev; lower panel…pZP2_TT_fw + pZP2_TT_rev.

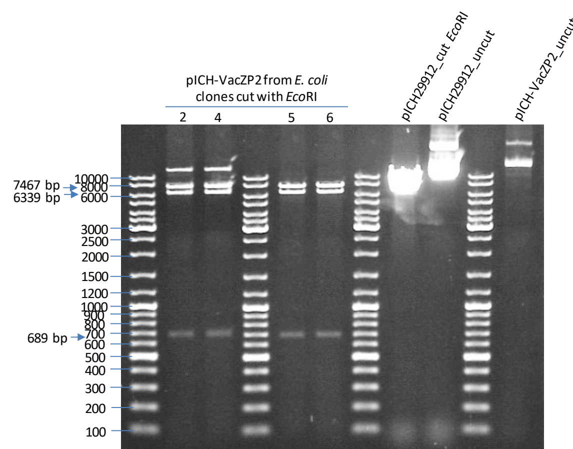


Figure 11: Restriction of pICH-VacZP2 from *E. coli* with *Eco*RI of clone 2-6 (lane 1-4). 5,6…pICH29912; 7…pICH-VacZP2 clone 2

$_{\Delta}$ZP2$_{only}$P

To obtain $_{\Delta}$*pZP2*$_{only}$*P* PCR was conducted with primer pZP2_pICH31120f and pZP2_pICH31120r with pICH-VacZP2 as template (Figure 13A). PCR product was cloned blunt end into pJET1.2 (Thermo Fischer™) and transformed into *E. coli* TG1 (Figure 12).

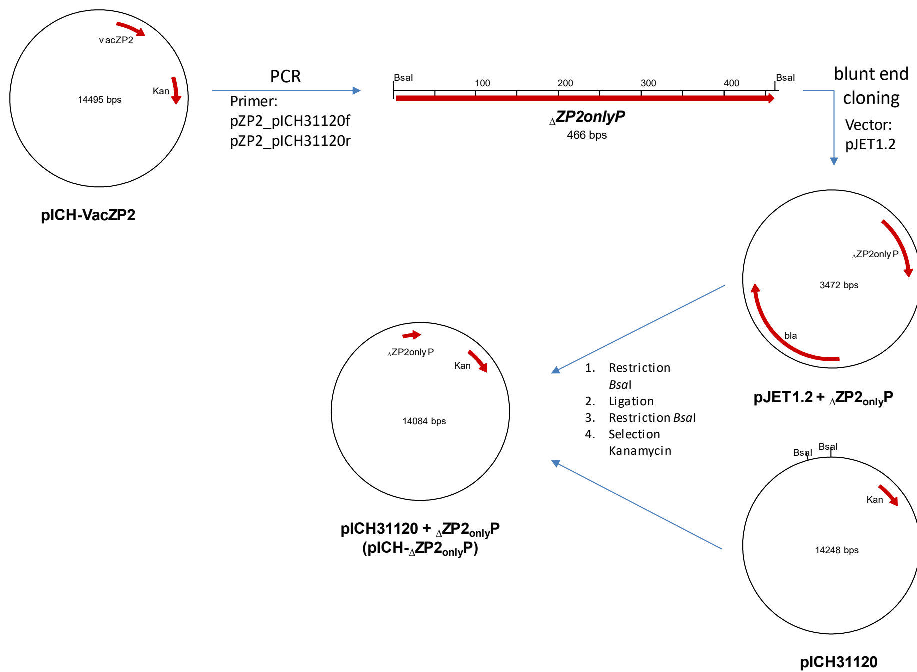


Figure 12: Overview of cloning steps to pICH-$_{\Delta}$ZP2$_{only}$P. bla…beta-lactamase gene for ampicillin resistance; km…*NPT*II gene for kanamycin resistance.

After selection on ampicillin positive clones were identified by colony-PCR (Table 7) with pJET1.2_fw and pJET1.2_rev primer (Figure 13B). The pJET1.2 + $_{\Delta}$pZP2$_{only}$P plasmid from clone 4 was positively verified in a sequence alignment (data not shown). $_{\Delta}$pZP2$_{only}$P was cloned into pICH31120 via *Bsa*I restriction sites (Engler et al. 2008). Nine clones selected on kanamycin were tested in a colony-PCR (Table 7). All clones were positive and the pICH-$_{\Delta}$pZP2$_{only}$P plasmid from clone 6 was used for transformation of A.t. ICF320 (Figure 13C).

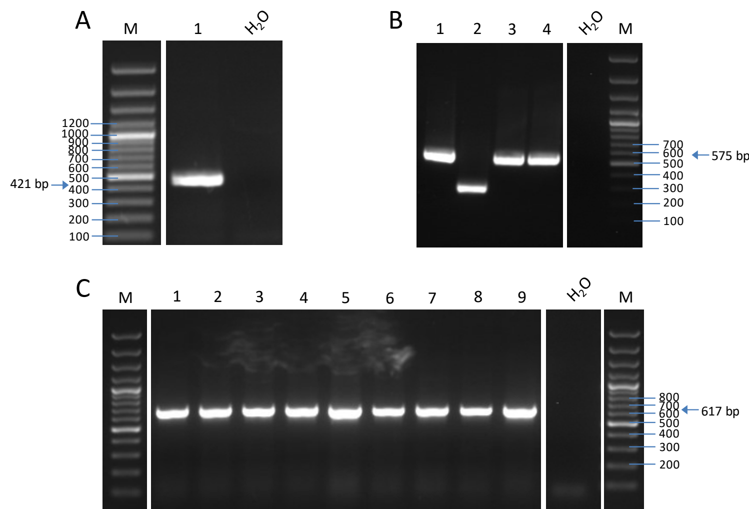


Figure 13: Verification of transforming *E. coli* with pICH-VacZP2. (A) Control PCR of pICH-VacZP2 with primer pZP2_pICH31120f + pZP2_pICH31120r leading to $_{\Delta}$pZP2$_{only}$P (421 bp). (B) Colony-PCR of *E. coli* TG1 transformed with pJET + $_{\Delta}$pZP2$_{only}$P. 1-4…clone 1-4. (C)

Colony-PCR of *E. coli* TG1 transformed with pICH-$_{\Delta}$pZP2$_{only}$P. 1-9…clone 1-9. H_2O…as template. M…DNA Ladder marker.

VacZP3-P

The fusion gene was synthesized by Eurofins (Ebersberg, Germany) and delivered in the plasmid pUC57 + VacZP3$_{BsaI}$. To obtain a functional *Bsa*I restriction site *vacZP3-P* was amplified with BsaI_repair_fw and BsaI_repair_rev (Table 9). The PCR product was detected in an agarose gel (Figure 15A) cloned blunt end into pJET1.2 plasmid, which resulted in pJET1.2 + VacZP3-P (Figure 14). This vector was transformed into *E. coli* TG1 and positive clones were identified by colony PCR (Figure 15B) with primer pJET1.2_fw and pJET1.2_rev (Table 9).

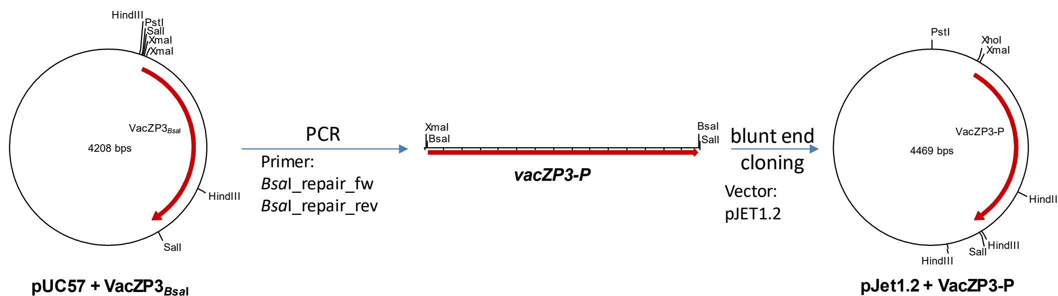


Figure 14: Cloning *vacZP3-P* into pJET1.2 gaining a functional *Bsa*I restriction site.

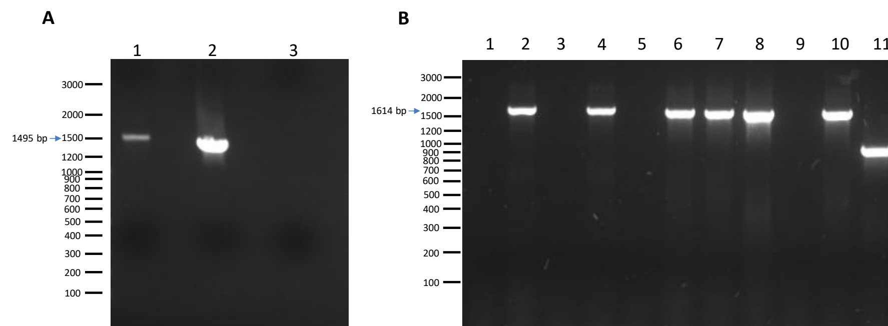


Figure 15: Verification of transforming *E. coli* with pUC57 + VacZP3$_{BsaI}$ and pJET + VacZP3-P. (A) PCR of pUC57 + VacZP3$_{BsaI}$ with primer BsaI_repair_fw + BsaI_repair_rev (5 µM). 1…1 µl PCR product. 2…9 µl PCR product. 3…H_2O negative control. (B) Colony-PCR with primer pJET1.2_fw and pJET1.2_rev on *E. coli* TG1 transformed with pJET + VacZP3-P. 1-11…clone 1 –11. Expected PCR Product: 1614 bp

Integrity of the plasmid pJET1.2 + VacZP3-P was verified by restriction with *Pst*I and *Xma*I to 4106 and 363 bp (Figure 16). PCR-mediated 3’ *Bsa*I site Integration (Figure 14) was tested by *Hind*III restriction to 271, 396 and 3802 bp fragments.

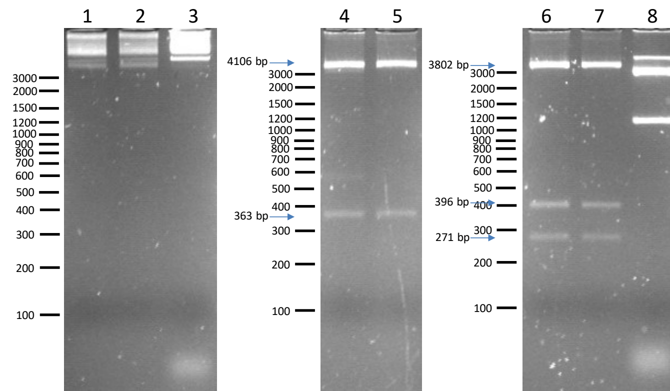


Figure 16: Restriction of pJET1.2 + VacZP3-P clone 2 and 6. 1,2…uncut plasmid; 3…uncut pUC57 + VacZP3$_{BsaI}$; 4,5…plasmid clone 2 and 6 cut with *Pst*I and *Xma*I; 6,7…plasmid clone 2 and 6 cut with *Hind*III; 8…pUC57 + VacZP3$_{BsaI}$ cut with *Hind*III

The fusion gene (Figure 5B) was inserted into the binary transformation vector pARC (Figure 5A) by *Xma*I and *Sma*I restriction sites from pJET1.2 + VacZP3-P and selected by streptomycin/spectinomycin (Figure 18A). Clones were tested by colony-PCR for ZP3 antigen (749 bp) and *vacZP3-P* (1489 bp) coding region with primer pZP3_Tabak_fw + pZP3_Tabak_rev and BsaI_repair_fw + BsaI_repair_rev, respectively (Table 9). All tested clones were positive (Figure 17) for both tests.

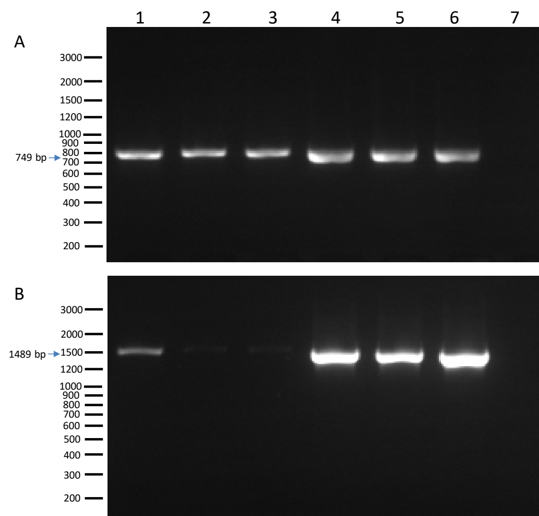


Figure 17: Colony-PCR of pARC- VacZP3-P transformed *E. coli* TG1. (A) Test on ZP3 antigen coding sequence with pZP3_Tabak_fw + pZP3_Tabak_rev. (B) BsaI_repair_fw + BsaI_respair_rev. Spur 1-5…Klon 1-5; Spur 6…positiv Kontrolle; 7…H_2O.

For transient expression we cloned the fusion gene *vacZP3-P* in the vector pICH29912 (Figure 5C) by *Bsa*I sites (Engler et al. 2008; Figure 18B).

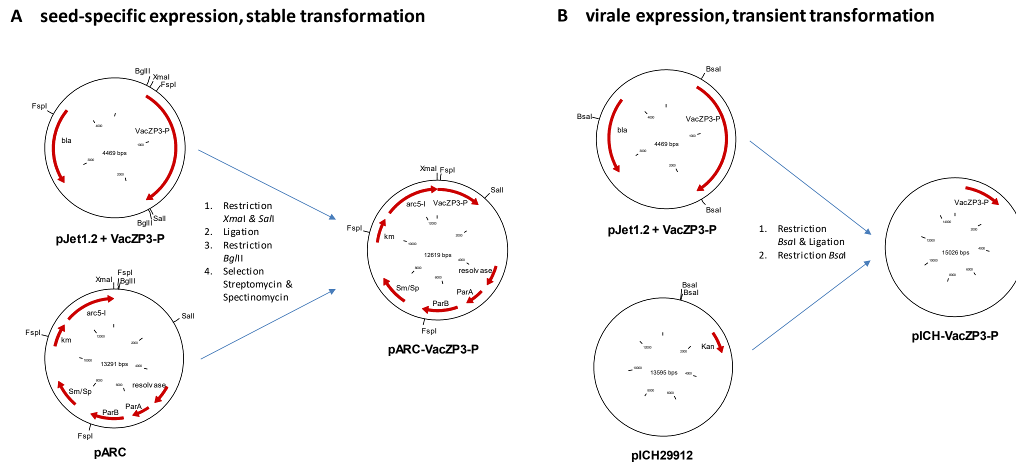


Figure 18: Overview of cloning strategies for VacZP3-P. (A) Seed-specific expression of VacZP3-P. arc5-I…*arcelin 5*-I promotor. ParA, ParB, resolvase…repressor for transposon activity; Sm/Sp…streptomycin/spectinomycin resistance gene. (B) Transient expression of VacZP3-P. km… neomycin phosphotransferase II (*NPT* II) for kanamycin resistance. bla…beta-lactamase gene for ampicillin resistance

Plasmid was transformed to *E. coli* TG1. Positive clones were identified by colony-PCR with primer Seq_pICH29912_fw and Seq_pICH29912_rev (Table 9). Plasmid from positive clone 10 (Figure 19A) carrying *vacZP3-P* was purified, sequenced and transformed to *A. tumefaciens* ICF320 strain. Kanamycin-selected clones were verified via colony-PCR, wheather they integrated *vacZP3-P* (1539 bp) with the ZP3 antigen (749 bp) (Figure 19B).

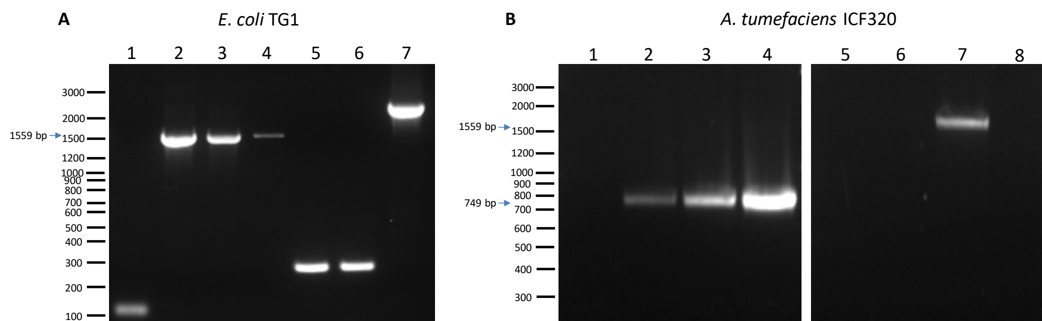


Figure 19: Colony-PCR for pICH-VacZP3-P transformed (A) *E. coli* TG1. 1-6…Clone 9-14, 7…positive control. Expected band: 1559 bp. (B) *A. tumefaciens* ICF320 clone 2 tested with i) lane 1-4…pZP3_Tabak_fw + pZP3_Tabak_rev and ii) lane 5-8… Seq_pICH29912_fw + Seq_pICH29912_rev. 1+5…H_2O negative control; 2+6…plasmid DNA diluted 1:100; 3+7…plasmid DNA diluted 1:20; 4+8…pICH-VacZP3-P plasmid DNA (lane 8 missing signal)

VacZP3-B

To obtain overexpression of VacZP3 in *E. coli* ER-targeting sequence has to be deleted out of the gene and cloned into an IPTG-induceable pET22b (Novagen) expression vector (Figure 20).

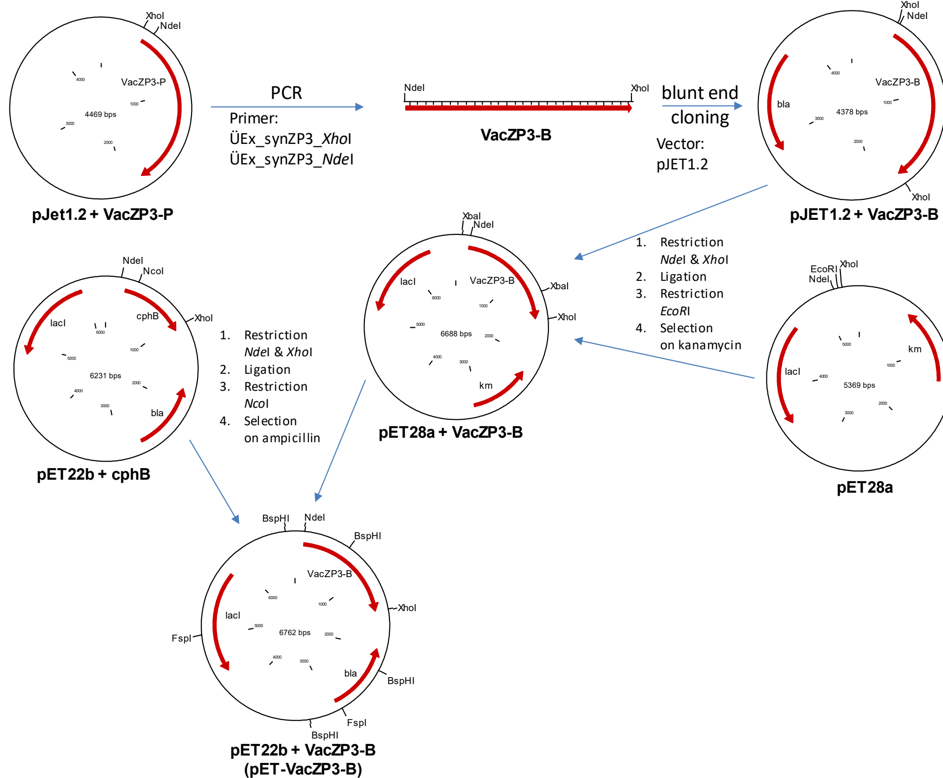


Figure 20: Overview of cloning steps to pET22b + VacZP3-B. bla…beta-lactamase gene for ampicillin resistance; km…*NPT*II gene for kanamycin resistance; lacI…lactose-inducible lac operon transcriptional repressor; cphB…cyanophycinase B gene.

Via PCR program 2 (Table 6) with the primer pair ÜEx_synZP3_*Xho*I and ÜEx_synZP3_*Nde*I (Table 9) *vacZP3-B* was created. The PCR product was ligated to vector pJET1.2 with CloneJET kit (Thermo Fischer™) and transformed to *E. coli* TG1. Positiv clones were identified by colony-PCR (Figure 21) with the primer pair pJET1.2_fw and pJET1.2_rev (Table 9). Clone 5 was pickt for further work.

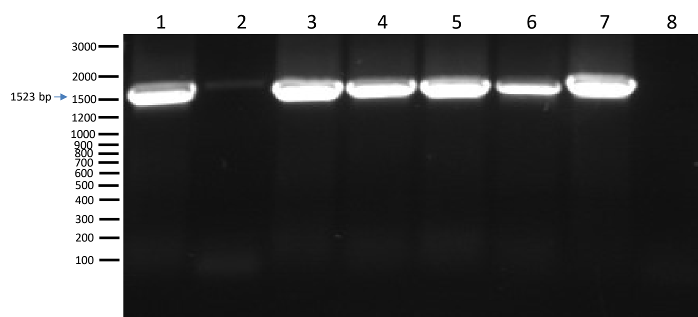


Figure 21: Colony-PCR with primers pJET1.2_fw + pJET1.2_rev of *E. coli* TG1 transformed with pJET1.2 + VacZP3-B. 1-6…Clone 1–6; 7…Positivkontrolle pJET1.2 + VacZP3-P, 8…H_2O negative control.

Because of the same antibiotica resistence of pJET1.2 and pET22b, an intermediate cloning step to pET28a via *Xho*I and *Nde*I restriction sites was necessary. False positive ligation products were linearized by *EcoR*I restriction. Positive clones growing on kanamycin were picked for further work. *VacZP3-B* was integrated to pET22b via *Xho*I and *Nde*I and false positive plasmids were linearized by *Nco*I. pET22b + VacZP3-B carrying *E.*

coli TG1 clones were selected on ampicillin and identified by colony-PCR with primer pair pET28a_fw and pET28a_rev (Table 9). Clone 8 was picked and the plasmid was, verified by *Fsp*I and *BspH*I restriction and subsequently sequenced. The plasmid was transformed to *E. coli* BL21 for overexpression.

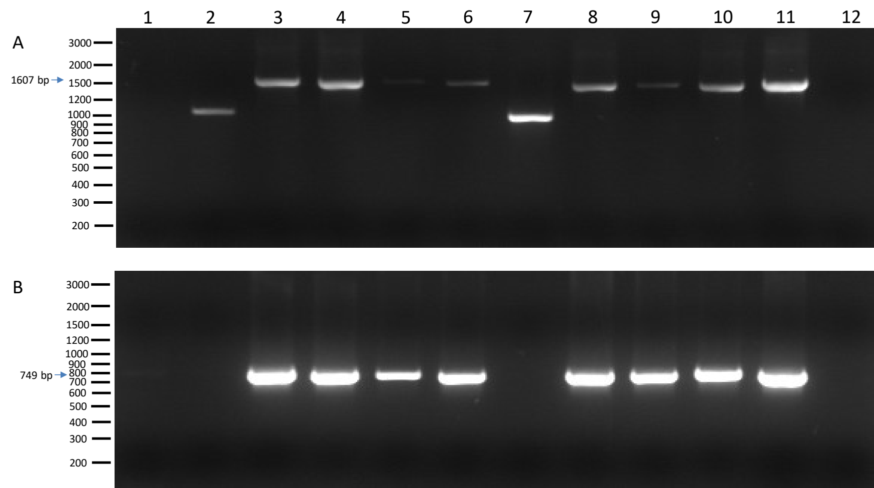


Figure 22: Colony-PCR of *E. coli* TG1 transformed with pET22b + VacZP3-B. (A) Primer pET28a_fw + pET28a_rev; PCR program 3. (B) Primer pZP3_Tabak_fw + pZP3_Tabak_rev; PCR program 4. 1-10…clone 1 – 10; 11…positive control pET28a + VacZP3-B. 12…H_2O negative control.

4.3 VacZP2

4.3.1 Stable expression of VacZP2

In order to identify the optimal production system for stable expression of VacZP2 we tested carrot cells and tobacco leaves and seeds as models.

Constitutive expression of VacZP2 in carrot calli

Carrot suspension cells were transformed using p35SS-VacZP2 (Figure 4A). 28 independent transgenic calli were identified via PCR (Table 3). Only 11 events show significant amounts of CTB with an average expression of 7 ng_{CTB} mg^{-1}_{TSP}. The maximum CTB content in calli was 20.7 ng_{CTB} mg^{-1}_{TSP} corresponding to 0.53 ng_{CTB} mg^{-1}_{DW} (

Figure 24A).

Table 3: Overview of expression levels in tobacco leaves, seeds and carrot cells. Level of expression was determined by anti-CTB ELISA. n. d. … not determined

Construct	Transformation rate [% of empty vector transformation]	Regenerated events	PCR positive events	ELISA positiv events	Average expression [% of TSP]	Highest expression level [% of TSP]
p35S-VacZP2 (carrot calli)	n. d.	n. d.	28	11	0.0007	0.00207
p35S-VacZP2 (tobacco leaf)	91.09	56	12	8	0.000038	0.000094
pARC-VacZP2 (tobacco seed)	95.33	79	29	24	0.096	0.248
pICH-VacZP2	n. d.	n. d.	n. d.	n. d.	4.0	5.2

Constitutive expression of VacZP2 in in tobacco

Stable leaf-disc transformation with p35S-VacZP2 showed no reduction in transformation rate in comparison to the empty vector (Table 3). After kanamycin selection, 12 PCR positive transformants were identified. The highest VacZP2 expression level in leaves was 0.94 ng_{CTB} mg^{-1}_{TSP} and 0.033 ng_{CTB} mg^{-1}_{DW}, respectively (

Figure 24A). The mean accumulation level is 0.38 ng_{CTB} $mg^{-1}_{TSP.}$

Seed specific expression of VacZP2 in tobacco

The *arcelin5*-I promotor from *Phaseolus vulgaris* (Goossens et al. 1994) was used to induce seed specific VacZP2 expression. *N. tabacum* Petite Havana SR1 was transformed with pARC-VacZP2 (Figure 4B). Out of 79 regenerated plants 24 putative VacZP2 producing events were obtained with unaltered development and development compared to the empty vector control pSingle35S (Table 3). The top event 59 reached accumulation levels up to 2479 ng_{CTB} mg^{-1}_{TSP} in ripe T_0 seeds corresponding to 0.24 μg_{CTB} mg^{-1}_{DW} (

Figure 24). Nine events produced more than 50% of the CTB identified in 59, but most of the events were under 1 μg_{CTB} mg^{-1}_{TSP} and 70 ng_{CTB} mg^{-1}_{DW}, respectively (

Figure 24B). Even at room temperature the amount of VacZP2 was stable in seeds for at least 18 weeks (

Figure 25B).

4.3.2 Transient expression of VacZP2 in N. benthamiana

For transient expression *Nicotiana benthamiana* plants were vacuum infiltrated with *Agrobacterium tumefaciens* carrying pICH-VacZP2 (Figure 4C). After 7 dpi leaves showed dry areas and bleached. This phenotype increased rapidly, but more frequently in plants infiltrated with the empty vector (Figure 23).

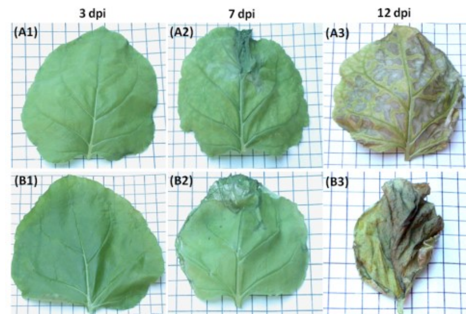


Figure 23: Leaves of *N. benthamiana* infiltrated with *A. tumefaciens* ICF320 carrying (A) pICH-VacZP2 and (B) empty pICH29912 vector, (1) 4 dpi (2) 7 dpi and (3) 12 dpi.

The content of VacZP2 in the freeze-dried leaf material was analyzed via anti-CTB ELISA. A significant increase of CTB was observed at the 9th dpi (

Figure 26). High variation was detected between the three independent experiments. Though no significant accumulation peak could be determined, all plant material for VacZP2 production was harvested at day 9. The average VacZP2 accumulation was 4 % per TSP (Table 3), which equals 1 µg VacZP2 per mg_{DW} in *N. benthamiana*. The second repetition of the experiment featured the highest accumulation of CTB with 52 $µg_{CTB}$ mg^{-1}_{TSP} and 1.2 $µg_{CTB}$ mg^{-1}_{DW} in a pool sample from the leaves of 30 plants (

Figure 24A). Tobacco leaves infiltrated with pICH-VacZP2 were freeze-dried and stored at room temperature in the dark. Storage for 35 weeks of storage did not significantly reduce the CTB content (

Figure 25A). After 74 weeks a significant decrease was detected.

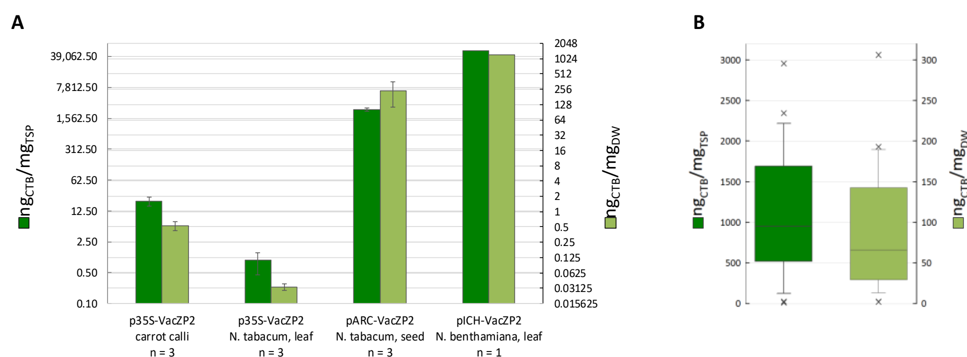


Figure 24: CTB concentration in different expression systems in relation to total soluble protein (TSP; dark green) and dry weight (DW; light green), respectively. (A) Top producing events of carrot cell suspension, constitutive 35S, seed-specific and transient expression system, respectively. Error bars show standard deviation. Bar for transient expression show a pool of 30 plants. (B) pARC-VacZP2 transformed T_0

tobacco seeds displayed in a boxplot (only CTB producing events integrated, n = 24).

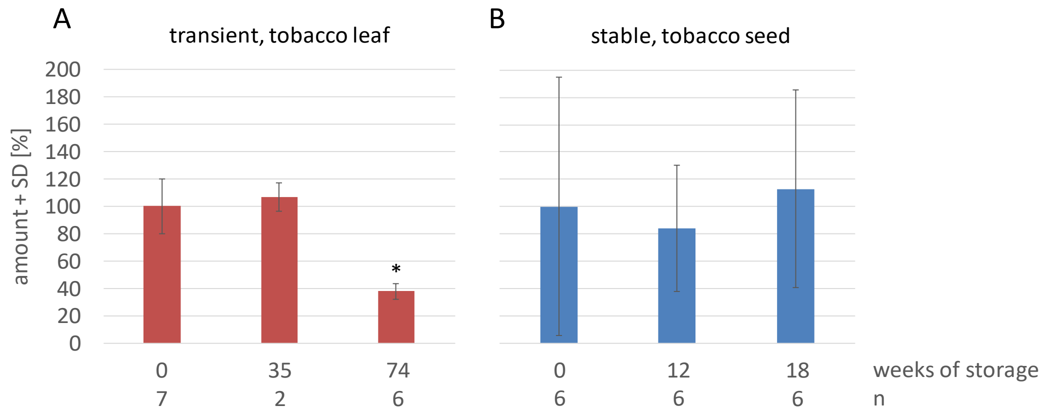


Figure 25: Stability of VacZP2 during storage in plant tissue. (A) Content of VacZP2 in transiently transformed, freeze-dried tobacco leaves in relation to 0 weeks of storage (red bars). (B) Content of VacZP2 in tobacco seeds in relation to 0 weeks of storage (blue bars). Error bars show standard deviation. Asterisk indicates a significant difference ($p < 0.05$).

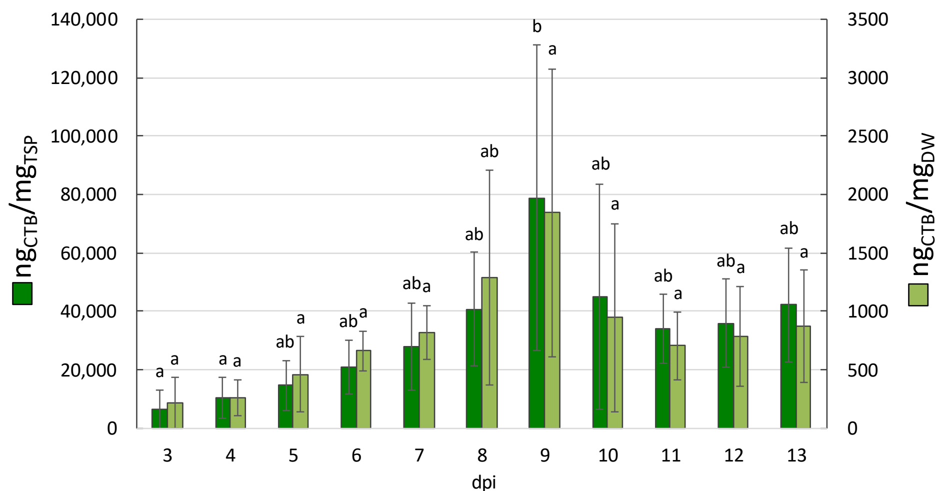


Figure 26: Accumulation of VacZP2 in *N. benthamiana* plants after agrobacterium-mediated transformation of pICH-VacZP2 (n = 3). All measurements by anti-CTB sandwich ELISA and corrected with values of NIC. Asterisk shows a significant difference ($p < 0.05$). Dark green columns refer to left axis. Light green columns refer to right axis.

4.3.3 Glycosylation and Stability of VacZP2

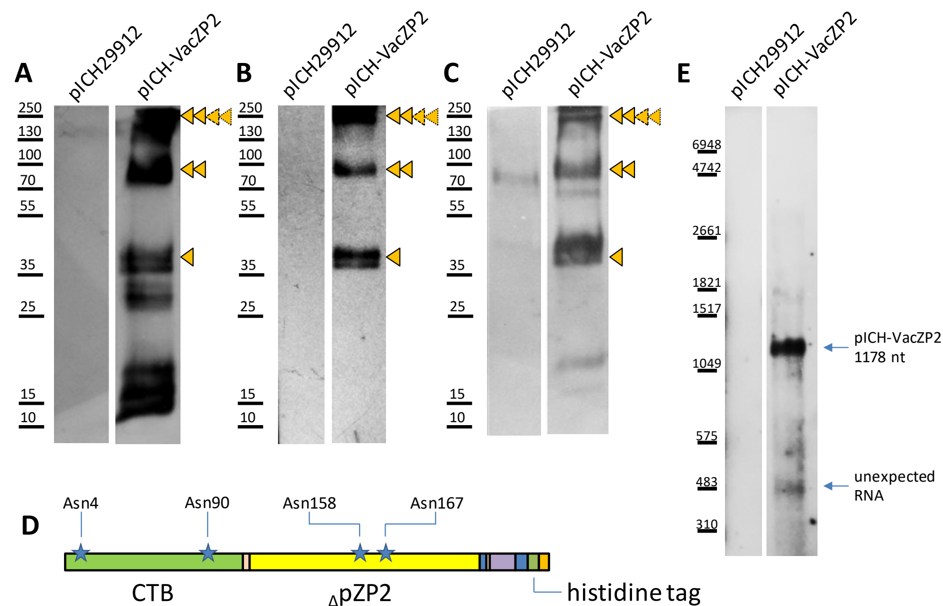


Figure 27: Detection of glycosylated VacZP2. (A) Anti-CTB Western Blot. (B) Anti-histidine$_6$ Western Blot. (C) Concanavalin A Western Blot. Samples are elution fractions after Ni-NTA purification. ◁...potential monomer of VacZP2. ◁◁...potential dimers of VacZP2. ◁◁◁◁...potential multimers$_{2<n\geq5}$ VacZP2. (D) Schematic representation of VacZP2. Stars show potential N-glycosylation sites. (E) Northern blot analysis of 2 µg RNA, isolated from *N. benthamiana* leaves, using a CTB probe. Lane 1: RNA from empty MagnICON™ vector in *N. benthamiana*. Lane 2: RNA from *N. benthamiana* leaves transformed with pICH-VacZP2 (expected mRNA: 1178 bp).

Based on the high expression level of VacZP2, the MagnICON system was chosen as production platform. To test the quality of VacZP2 after harvest the purified VacZP2 was separated via 12 % SDS-PAGE and an anti-CTB, anti-histidine or Concanavalin A Western blot analysis was performed (Figure 27ABC). Two proteins of around 35 and 41 kDa were detected in the elution fractions of Ni-NTA column in all three blots (one triangle). These proteins are slightly bigger than the calculated 32.2 kDa for the monomeric, non-glycosylated VacZP2 using the ProtParam tool (expasy.com). Presuming 3 kDa per *N*-glycan, protein masses between 35 and 44 kDa were expected for a glycosylated VacZP2 monomer, corresponding to the number of potential maximal four *N*-glycosylation sites (Figure 27D).

In addition, various bands between 70 and 250 kDa were detected in all three Western blots (Figure 27ABC), which could represent oligomers of VacZP2 oligomerized base on the CTB domain.

Lower bands between 12 and 18 kDa are detectable in anti-CTB and Concanavalin A Western blot analysis (Figure 27AC), but not in the anti-histidine Western blot analysis (Figure 27B). These signals correspond to monomeric (11.6 kDa), non-, once (15 kDa) and twice glycosylated CTB (18 kDa), respectively, if a glycan mass of 3 kDa is assumed (Mishra et al. 2006). Bands between 25 and 35 kDa may represent dimers of the CTB molecules.

An RNA corresponding to the length of the smallest viral RNA of *vacZP2* (1178 nt) was detected in Northern blot analysis using CTB as probe (Figure 27E). In addition, a small RNA was detected at 483 bp. The analysis of the DNA sequence downstream of the CTB coding region did not reveal any specific transcriptional termination signal (Suppl. 1).

4.3.4 Purification and Quantification of VacZP2

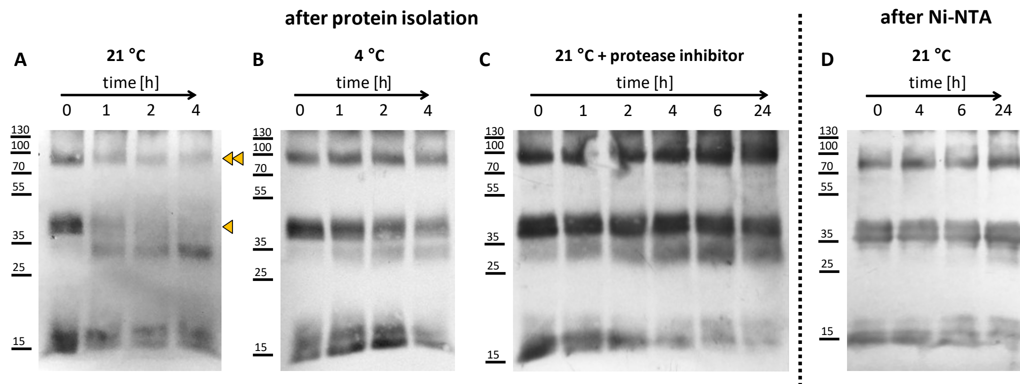


Figure 28: Stability of VacZP2 in tobacco leaf extracts over time. Detection using anti-CTB Western blot analysis. (A) 5 µl protein extract incubated at 21 °C. (B) 5 µl protein extract incubated at 4 °C. (C) 2.5 µl protein extract incubated at 21 °C with protease inhibitor mix. (D) 4.5 µg total protein of elution fraction of VacZP2 in 1x PBS (pH 7.2) after Ni-NTA purification. ◁…potential monomeric VacZP2. ◁◁…potential dimer of VacZP2.

Potentially monomeric and dimeric VacZP2 decreases in leaf extract at room temperature (Figure 28A). This effect is alleviated when TSP extract is stored at 4 °C (Figure 28B). A complete stabilization is achieved by addition of a protease inhibitor mix (Figure 28C) or after purification via a Ni-NTA column (Figure 28D). The signal for putative CTB oligomers became stronger during incubation (Figure 28A, B, D). Signals between 12 and 18 kDa potentially representing CTB are present immediately after TSP extraction independent of protease inhibitor addition or cooling.

Since free CTB molecules are still present in the purified vaccine (Figure 28D), they lead to an overestimation of antigen in the anti-CTB ELISA. To estimate the content of VacZP2, we calculated the ratio of free CTB molecules in TSP by densitometric analysis of Western blot signals via image processing (ImageJ) (Suppl. 5). VacZP2 accounts for 88% of the full fusion protein in the vaccine.

4.3.5 *Transient expression of $_{\Delta}$pZP2$_{only}$P*

The Concanavalin A Western blot analysis of VacZP2 (Figure 27) shows glycosylation of the whole fusion protein, nevertheless it is not possible to distinguish between glycosylations of $_{\Delta}$pZP2 and the rest of the fusion protein. To be ascertained whether $_{\Delta}$pZP2 or CTB is glycosylated, $_{\Delta}$pZP2 alone was integrated into the vector pICH31120 ($_{\Delta}$ZP2$_{only}$P, Figure 4F) and transiently expressed in *N. benthamiana.* In the anti-histidine Western blot analysis and Coomassie stained SDS-PAGE a signal with the expected size of $_{\Delta}$pZP2$_{only}$P between 17 and 20 kDa was identified (Figure 29AB). This signal was also found in the lectin blot, which proofs that at least one glycosylation site is recognized in $_{\Delta}$pZP2 (Figure 29C). The signal in the anti-histidine western blot between 17 and 20 kDa is very strong and could be a double signal of non- and once glycosylated monomer. Two signals around 35 kDa might be a result of dimerization. A signal at 11 kDa could be a hint for proteolytical sensitivity of $_{\Delta}$ZP2$_{only}$P.

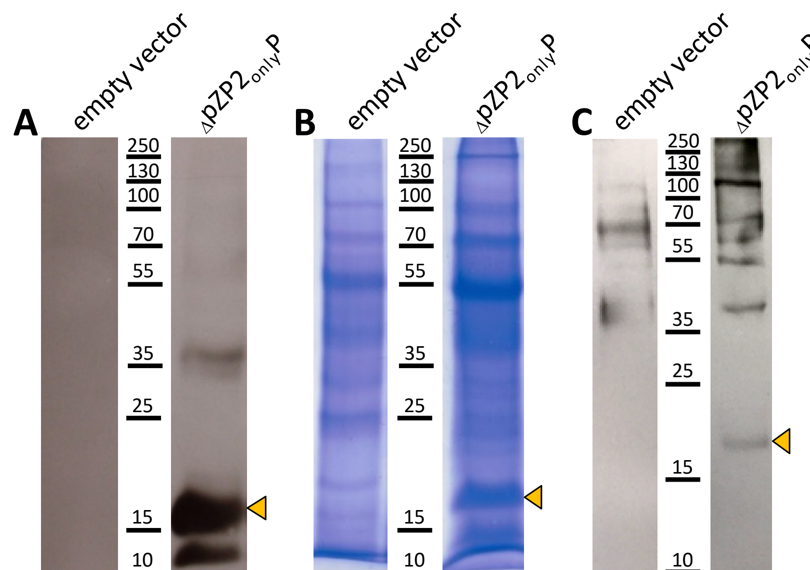


Figure 29: Detection of $_{\Delta}$pZP2$_{only}$P after expression via MagnICON. (A) Anti-histidine Western blot analysis. (B) Coomassie stained 12 % SDS PAGE. (C) Concanavalin A conjugated POD detects high mannose residues. ◁…potential $_{\Delta}$pZP2$_{only}$P. Unglycosylated calculated mass is 16.7 kDa.

4.3.6 Animal trial

Strong immune response in mice

The immunogenicity of VacZP2 was tested via three parenteral applications of 264 µg fusion protein per mouse. Sera of all bleedings were equally diluted 1:50,000 and measured in microtiter plates coated with the vaccine (Figure 30A). After subcutaneous priming (day 2) no significant immune response was observed in the second bleeding (day 21), when the sera diluted 1:50,000 measured against the complete VacZP2 vaccine. But a lower dilution of 1:100 shows a significant increase after priming with VacZP2, which was approx. 7-fold higher with adjuvant (Suppl. 7). After first subcutaneous booster injection of 264 µg vaccine (day 26) we observed a significant increase of antibodies in the third bleeding (day 37), no matter whether VacZP2 was delivered with or without adjuvant. A second booster with 264 µg vaccine raises the immune response significantly.

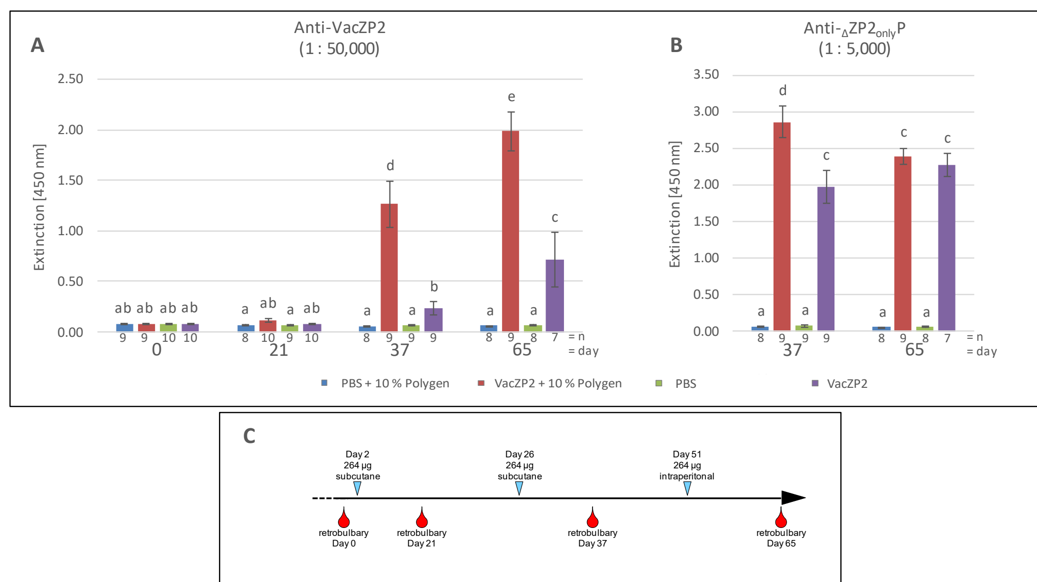


Figure 30: (A) Detection of antibodies against VacZP2 in FvB/NcrL mice. Sera were diluted 1:50,000. (B) Detection of antibodies against $_{\Delta}$pZP2$_{only}$P in FvB/NcrL mice. Same sera like in A, but diluted 1:5,000. Error bars show standard deviation. Significance classes calculated by post-hoc Tukey-HSD ($p > 0.05$). (C): Timeline of the immunization schedule. Bleeding (red drops) and vaccination (blue triangles) regime of FvB/NcrL mice during the animal trial.

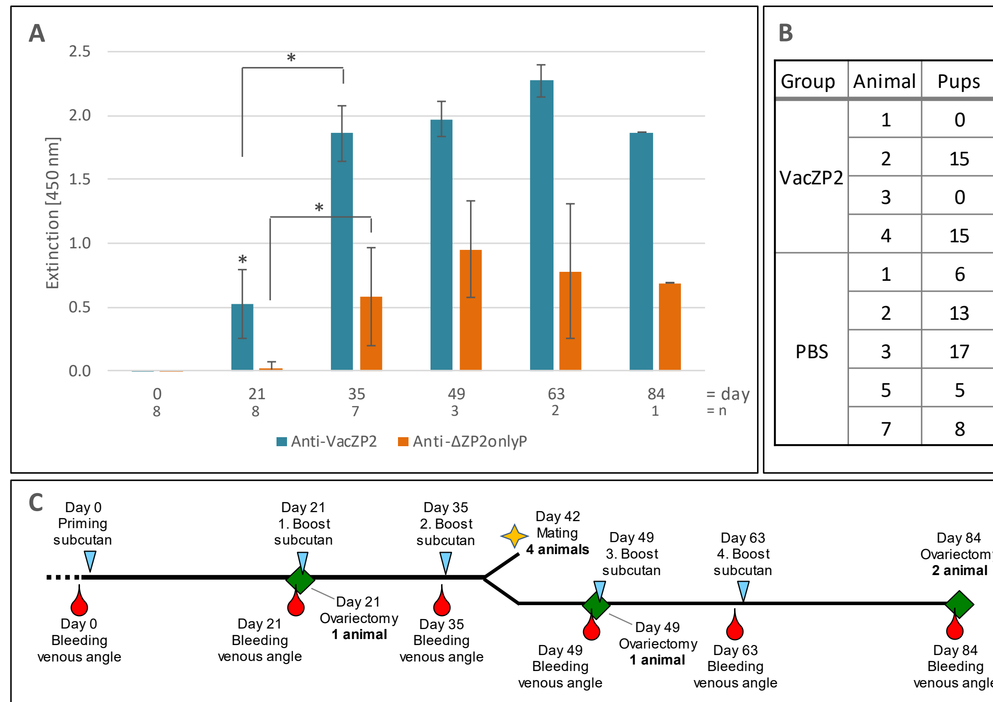
To analyze whether the response is only connected to CTB and TT or also to the $_{\Delta}$pZP2 domain, microtiter plates are coated with $_{\Delta}$pZP2$_{only}$P, a plant-derived $_{\Delta}$pZP2 peptide. The sera of all immunized animals were diluted 1:5,000 and measured in this ELISA. After priming, antigen specific $_{\Delta}$pZP2 antibodies showed a significant increase, when applied with adjuvant (Figure 30B). Second boostering resulted in no further increase of the immune response.

Hamster infertile after VacZP2 vaccination

Eight animals of golden hamster were immunized three times with 20 µg VacZP2 + 10 % Polygen. Antibody titer as well as against the fusion protein VacZP2 and the $_{\Delta}$pZP2 antigen domain by coating the plant-made $_{\Delta}$pZP2$_{only}$P petide to the ELISA plate. After priming, a significant increase of antibodies against VacZP2 were visible (Figure 31A). Antibody titer

significantly increased after first boost against fusion protein and antigen domain. Subsequent booster injections did not increase the titer.

Four of eight animals were mated with males of proven fertility. Two animals had no litter (Figure 31B). In the control group, all animals were pregnant. The two spayed females from the VacZP2 group were mated again at day 63 and day 59 without additional vaccination and each gave birth to 16 pups (data not shown).



Group	Animal	Pups
VacZP2	1	0
	2	15
	3	0
	4	15
PBS	1	6
	2	13
	3	17
	5	5
	7	8

Figure 31: (A) Detection of anti-VacZP2 and anti-ΔpZP2$_{only}$P antibodies after vaccination of golden hamster with 20 µg VacZP2 + 10 % Polygen. Sera were diluted 1:1,000. Values were corrected by PBS group titer. Error bars show standard deviation. (B) Number of born pups after three immunizations. (C) Timeline of VacZP2 vaccination (blue triangles), bleedings (red drops), mating (yellow star) and ovariectomy (green diamond). Asterisk show significant difference ($p < 0.05$, Tukey-HSD)

The remaining four animals in each group were subsequently vaccinated with the same amounts and euthanized at day 84. Ovaries were surgically removed and histologically examined. The histological evaluation of the female reproductive tract (ovary, oviduct and uterus) on day 84 after the last protein application revealed no signs of inflammation in any animal under investigation. Corpora lutea as well as follicles in different stages of development were present on the ovaries of all animals, suggesting normal cyclicity.

4.4 VacZP3

4.4.1 Constitutive Expression of VacZP3-P in seeds

Tobacco

The transformation with pARC-VacZP3-P led to similar regeneration rates and phenotype of the transformands compared to the empty vector control. 16 events were selected for seed production. All events produced normal amounts of viable seeds except event 144 which produced a mixture of full and empty seeds which contained the highest amount of CTB (303 ng/mg TSP). For protein isolation seeds from ten capsules of each event were pooled and the CTB content was measured in an anti-CTB ELISA. The average accumulation of VacZP3-P in seeds of the top six events was 0.008 % / total soluble protein (TSP) and 9 ng/mg dry weight (DW) (Figure 33A).

In dry seeds the fusion protein was stable for over 84 weeks (Figure 34B).

Pea

Transformation of 750 seeds with pARC-VacZP3-P resulted in 30 positively tested transgenic plants, 23 of which were cultivated for seed production. No aberrant phenotype was observed during in-vitro cultivation. Samples were taken from at least four randomly chosen seeds of each event and tested in the anti-CTB ELISA, except event 17, which only produced one seed. Seeds, where no CTB could be detected, were defined as non-producer and sorted out. We received 18 CTB producing events. 13 of all producing events exhibited less than 50% of the CTB/TSP accumulated in the top event 16 with 0.53 ng_{CTB} / mg_{TSP}^{-1} and 0.01 ng_{CTB} / mg_{DW}^{-1} on average (Figure 33B). The top producing seed of event 16 accumulated 9.3 x 10^{-5} % $_{CTB}$ /TSP.

4.4.2 Transient expression of VacZP3-P in N. benthamiana leaves

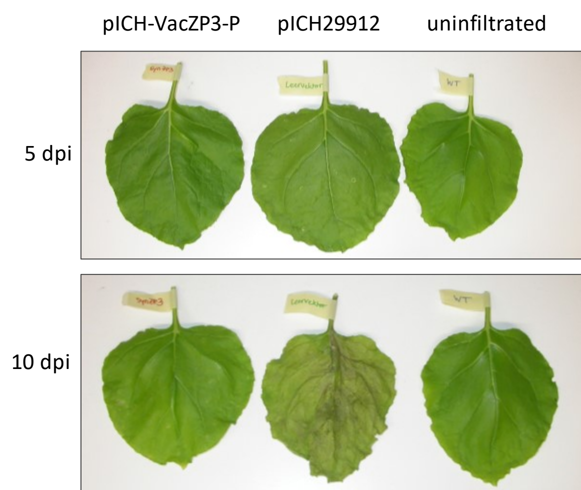


Figure 32: Phenotypical changes of pICH-VacZP3 infiltrated *N. benthamiana* leaves after 5 and 10 days post infiltration (dpi).

Eleven *N. benthamiana* plants were vacuum infiltrated with *A. tumefaciens* ICF320 carrying plasmid VacZP3-P (Figure 5C) in minimal 3 repetitions. In order to determine the accumulation of VacZP3-P, minimal four single leaves with different developmental stages were harvested from 3 till 13 days post-infiltration (dpi) and freeze-dried. The CTB content

in the leaves was analyzed by an anti-CTB sandwich ELISA with commercially available CTB as standard. The maximal mean accumulation in leaves of 3.76 $\mu g_{CTB} \cdot mg_{TSP}^{-1}$ is reached at the 11th dpi, which is significantly different to day three and four (Figure 33C). pICH-VacZP3-P infiltrated leaves bleached and dried out. This effect was even stronger in leaves infiltrated with the empty vector (Figure 32). Therefore, in the vaccine production phase plants were harvested at 11th dpi.

According to the CTB detected per TSP the transient expression of VacZP3-P was approximately 12 times higher compared to tobacco seeds and over 4500 times higher compared to pea seeds both under control of the arcelin promoter.

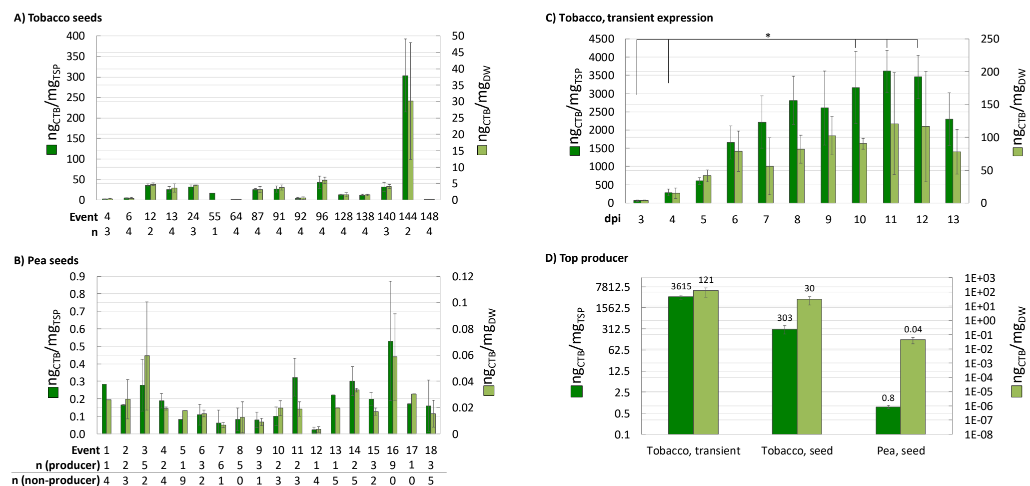


Figure 33: Accumulation of VacZP3-P in different plant expressions systems measured by anti-CTB ELISA (corrected by empty vector control). Dark green bars refer to left axis and light green bars to right axis, respectively. (A) VacZP3-P in pooled T1 tobacco seeds. (B) VacZP3-P in producing T1 pea seeds. Non-producers are excluded from the calculation. (C) VacZP3-P in tobacco leaves after transient expression. ($n_{dark\ green}$ = 5, $n_{light\ green}$ = 3). (D) Highest CTB concentrations of each expression system. Transient expression: values of 11th dpi already shown in C; Tobacco seed event 144; Pea seeds: Mean value of the five highest expressing seeds from event 16. Error bars show standard deviation. Numbers on top of each bar show mean value. Logarithmic scale. Asterisks show significance ($p < 0.05$; Tukey-HSD).

After freeze-drying, the CTB content was not reduced significantly (data not shown). Also, storage of freeze-dried tobacco leaf material did not decrease the CTB content for at least 21 weeks at room temperature in the dark. After 68 weeks of storage a significant reduction of about 40 % was observed (Figure 34A).

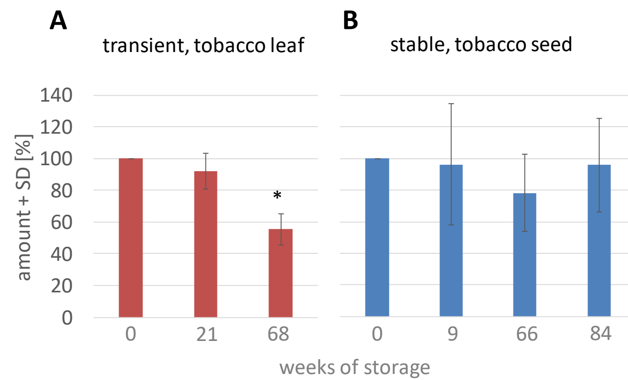


Figure 34: Stability of VacZP3-P during storage in plant tissue. (A) Content of VacZP3-P in transiently transformed, freeze-dried tobacco leaves in relation to 0 weeks of storage (red bars, n = 7) (B) Content of VacZP3-P in tobacco seeds in relation to 0 weeks of storage (blue bars, n = 7). Error bars show standard deviation. Asterisk indicates a significant difference ($p < 0.05$).

4.4.3 Glycosylation and assembly of VacZP3-P

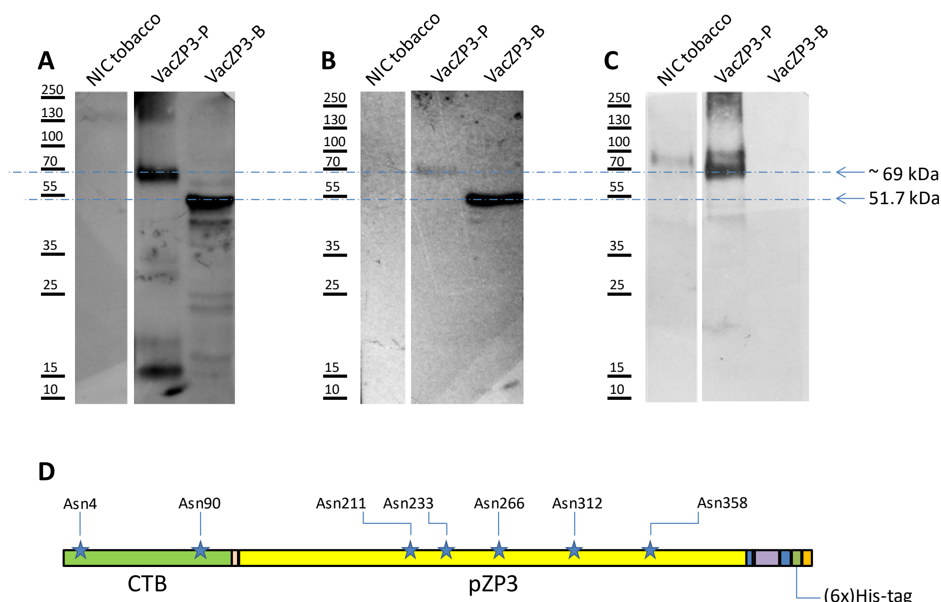


Figure 35: Detection of VacZP3-P via Western Blot with different primary antibodies. (A) Anti-CTB. (B) Anti-histidine tag. (C) Concanavalin A. Samples of VacZP3-P and VacZP3-B are elution fractions after Ni-NTA purification. (D) Schematic figure of VacZP3-P monomeric fusion protein. Stars show potential N-glycosylation sites.

Transiently expressed VacZP3-P was detected in Western blots with antibodies binding either to the N- (anti-CTB, Figure 35A) or C- (Anti-histidine, Figure 35B) terminus of the fusion protein. *E. coli* derived VacZP3-B served as positive control. Based on the data published for CTB, where N-glycosylation varied between 1.5 (Matoba et al. 2009), 2 (Hamorsky et al. 2015) and 3 kDa (Mishra et al. 2006), we calculated each putative N-glycosylation (Asn-X-Thr/Ser) of the fusion protein with a mass increase of 3 kDa. According to this assumption and dependent on the number of glycosylation sites occupied the plant made VacZP3-P should have a size between 55 and 73 kDa. Only one

band at 69 kDa could be detected in anti-CTB, anti-His and Concanavalin A Western blots. This 69 kDa band was not visible, when VacZP3 was bacterially expressed, hence homogenous, possibly incomplete glycosylation or lighter N-glycans at the plant-made protein can be assumed (Figure 35).

Signals above 130 kDa detected in anti-CTB and Concanavalin A Western blot analysis might refer to glycosylated VacZP3-P dimers with an expected mass between 121 and 145 kDa (Suppl. 2C, Figure 35A). Due to the fusion to CTB VacZP3-P should assemble to a pentamer (Kwon and Daniell 2016; Yasuda et al. 1998). Nevertheless, even with lower acrylamide concentrations (6%) and a native protein separation, the precise determination of molecular mass of the expected multimers / pentamers were not possible (Suppl. 2C). However, the formation of pentamers could be proven by a GM1 ELISA of crude protein VacZP3-P extracts (

Suppl. **3**A) since only the pentamers bind to the mono-sialoganglioside GM1 receptor (Wolf et al. 1981; Dakterzada et al. 2012).

4.4.4 Quantification of VacZP3-P

The amount of fusion protein was quantified by an anti-CTB ELISA. In order to identify the quantity of intact fusion protein in the vaccine, potential degradation of the fusion protein was analyzed via Western blot analysis. Degradation products smaller than the VacZP3-P monomer was observed via anti-CTB Western blot analysis directly after isolation (Figure 36A). This phenomenon could not be avoided by the addition of protease inhibitors (Figure 36C) or change of the isolation buffer (Davoodi-Semiromi et al. 2010) (Suppl. 4). The strongest signals were between 10 and 20 kDa in the anti-CTB Western blot analysis. These signals match the size of non-, once and twice glycosylated CTB monomers, when a glycan mass of 3 kDa is assumed (Mishra et al. 2006). Northern blot analysis with a CTB probe gave a signal for the smallest viral mRNA of *vacZP3-P* (1709 nt) and a very weak for an around 483 nt long mRNA (Figure 36G), which is longer than the coding region for CTB from ATG to the GPGP linker (381 bp). Untranlated regions at the 5’ and 3’ end of the vacZP3-P gene could cause this size. But no transcriptional stop or poly a signal was detected (Suppl. 6) in the sequence at approx. 380 bp, which would explain the occurrence of the small CTB.

A general degradation of VacZP3-B was observed directly after isolation from *E. coli* under denaturating (data not shown) and native conditions in anti-CTB Western blot analysis (Figure 35A). The degradation products match most of those detected in the plant taking the lack of glycosylation into account, however, a degradation product matching the size of CTB could not be detected. The anti-CTB Northern blot analysis with VacZP3-B detected no smaller mRNA fitting to CTB monomer molecules (Figure 36G).

Incubation of VacZP3-P monomers isolated either from plants or bacteria in crude extracts of untransformed *N. benthamiana* at room temperature reproductively (n=3) resulted in degradation of the proteins detected via anti-CTB Western blot analysis (Figure 36AE). With cooling or addition of protease inhibitor mix the degradation of VacZP3-P could be reduced (Figure 36BC) and completely vanished after purification via Ni-NTA column (Figure 36D). An increase of degradation products of VacZP3-P during incubation was not observed.

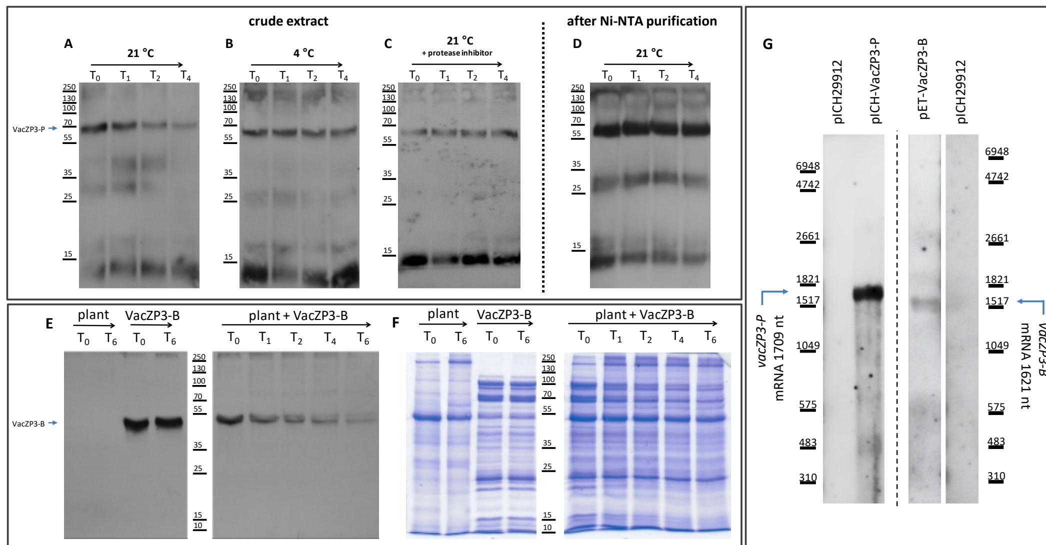


Figure 36: Stability of VacZP3. (A – D) Anti-CTB Western Blot of VacZP3-P in tobacco leaf extract at (A) 21 °C, (B) 4 °C and (C) 21 °C with protease inhibitor mix, respectively. (D) Incubation of VacZP3-P in 1x PBS (pH 7.2) after Ni-NTA purification. (E) Anti-CTB Western blot analysis with 10 µg of VacZP3-B incubated in 10 µg tobacco crude extract at room temperature for maximum 6 hours. (F) Same loading like E, but gel stained with Coomassie. (G) Anti-CTB Northern blot analysis of 5 µg RNA from *N. benthamiana* leaves and 2 µg from *E. coli* transformed with pICH-VacZP3-P and pET-VacZP3-B, respectively.

Since free CTB molecules in the extracts lead to an overestimation of VacZP3-P in the Anti-CTB ELISA we determined the amount of free CTB via densitometric analysis. According to these results the actual VacZP3-P content in the plant is 8% less then estimated via the Anti-CTB ELISA (Suppl. 5)

4.4.5 Plant-made VacZP3-P is more immunogenic than the bacterial control

Three groups of ten mice were vaccinated three times with two, seven and 21 µg VacZP3-P, respectively. Animals were retrobulbary bled once before and three times several days after each treatment (Figure 37C). A significant increase of the antibody titer was observed after priming with two, seven and 21 µg VacZP3-P (Figure 37A). After the first boost, all VacZP3-P vaccinated mice more than doubled the amount of antibodies in their blood and were significantly different to the PBS control group. Across the experiment there is no significant difference in antibody titer between 2 and 7 µg VacZP3-P vaccination. After two boosts the group, which received 21 µg VacZP3-P exhibited an 18 % higher titer than the two other VacZP3-P groups. The bacterial fusion protein TT-KK-ZP3 (Gupta et al. 2013) was used to verify the responsiveness of the BALB/c mice to porcine ZP3 antigen. Antibodies against VacZP3-P were not able to bind to immobilized TT-KK-ZP3.

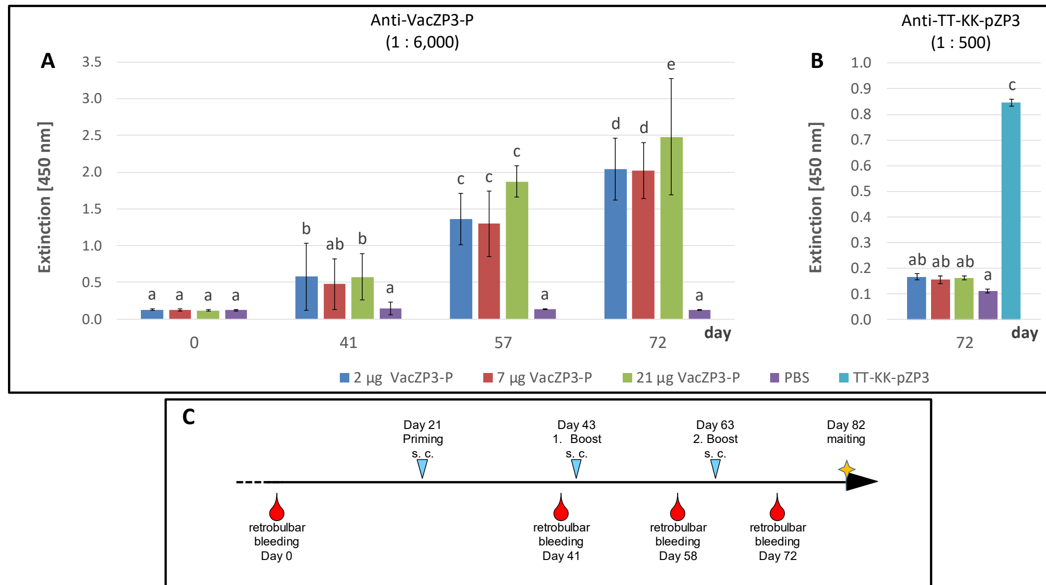


Figure 37: Antibody titer of vaccinated female BALB/c mice (n = 10 per group, 9 mice at day 72 receiving 2 and 7 µg VacZP3-P). All doses contained 10 % Polygen as adjuvant. Letters show homogenous subsets by Tukey-HSD. (A) All sera were diluted 1:6,000 and measured against VacZP3-P, coated on microtiter plates. (B) All sera diluted 1:500 and measured against bacterial TT-KK-pZP3 (Gupta et al. 2013), coated on microtiter plates. (C) Regime of vaccination. Blue triangles...time point of vaccination; Red drops...time point of bleeding.

On day 82 mice were mated with fertility proven males. A dosage-dependent litter reduction was observed (Table 4). The offspring in group four, which received 21 µg VacZP3-P, was reduced 43 % in comparison to the PBS control group, which had regular numbers of pups. Nevertheless, the reduction was not significant. Comparing plant and bacterial vaccine, VacZP3-P had a stronger, but not significant, influence on fertility. No correlation between antibody titer and sterility was observed.

Table 4: Mating studies with vaccinated BALB/c mice.

Group			Failed to conceive	Pups
1	PBS	+ 10 % Polygen	0 of 10	60
2	2 µg VacZP3-P	+ 10 % Polygen	0 of 10	53
3	7 µg VacZP3-P	+ 10 % Polygen	1 of 10	36
4	21 µg VacZP3-P	+ 10 % Polygen	3 of 9	26
5	25 µg TT-KK-pZP3	+ 10 % Polygen	1 of 10	42

5. Discussion

VacZP2 and VacZP3-P were detected in all plant expression systems. Tobacco seeds and transient expression exhibited high, table transformed carrot cells, tobacco leaves and pea seeds exhibited low accumulation levels. Promoter, stability, intracellular targeting and the host define the expression and accumulation level of the plant made pharmaceutical (Schillberg et al. 2005; Faye et al. 2005a; Stöger et al. 2002), which was the reason to test a variety of plant production platforms for expressing VacZP2 and VacZP3-P. To pick one of the listed, the stability, it includes the stability of the transcript at RNA level leading to an efficient translation to the protein and the resistance against endogenous proteases at protein level (Stöger et al. 2002). On the one hand it is defined in the nucleotide and amino acid sequence of the transgene, in the other hand at the plant host itself, owing specific transcription/translation features and specific proteases, which distinguish it to other plant species. The expression of scFv84.66 in different plant species under the control of the 35SS promotor exhibited a the variation in in tobacco, rice and tomato leaves (Stöger et al. 2002), documenting host-dependend accumulation level. Even the relatively minor difference of the genetic background in cultivars of one species could lead to different accumulation levels of a recombinant protein (Nausch et al. 2012a). It was necessary to experimentally test different plant expression systems to evaluate the optimal expression system in this study.

5.1 Expression systems

Carrot cells

VacZP2 transformed carrot cells (cv. Rotin) expressed up to to 0.53 $\mu g_{CTB}/g_{DW}$ under the control of the CaMV35S promoter. This result is very poor for carrot cell expression, but not alone with low yield in this setting (Mikschofsky et al. 2009c). Carrot is widely used in biotechnology and is capable of establishing high production. In 2012, it became the production platform of the first approved plant-made enzyme for human therapy against Gaucher disease (Protalix.com). Protalix uses a different setting for production. It cultivates the transgenic carrot cells in liquid suspension media, stores their glucocerebrosidase inside of vacuoles, and uses a different terminator (octopine synthase) and another translational enhancer element (Ω translational element). All these factors are different to our setting and could influence the outcome. Promoter, transgene and terminator have to be appropriate to each other and determine the expression level (Nagaya et al. 2010). Changing our settings of expression vector to this of Protalix could result in a higher accumulation level. Furthermore, the enhancer element could improve translational efficacy and resulting yield (Chen et al. 2001).

It could also be possible that the activity of metabolism is lower in calli on solid agarose than in liquid suspension media, because nutrient supply should be much better in liquid media (Ziv 1995). In consequence the synthesis of VacZP2 may be higher in carrot suspension culture. In regard to the storage compartment, a change of localizing VacZP2 to the vacuole might increase the yield. Protein sequestration in subcellular compartments is known to be a major factor to stabilize recombinant proteins in plants (Benchabane et al. 2008). Vacuoles host different specific enzymatic conditions, including protease composition and pH. E.g. complement factor 5a (C5a) accumulated better in vacuole of tobacco cells than in ER or apoplast (Nausch et al. 2012b). It could be a measure

to increase accumulation of VacZP2 in carrot cells, because of detected protease sensitivity of tobacco-derived VacZP2. It could also be an improvement strategy to use another cultivar of carrot than Rotin. Mikschofsky et al. 2009c has shown that an expression of VP60 in the cultivar Gelbe Futter exhibit a more than 2.5-fold increase in comparison to Rotin. Kalbina et al. 2011 used the carrot cultivar Karotan and Napoli and produced a Chlamydia trachomatis vaccine with up to 3 % of, which is 1449-times higher in comparison to our VacZP2 accumulation.

Tobacco leaf

VacZP2 was accumulated up to 0.000094 % of TSP in the top event. This detected amount is very low but showed in two independent measurements a clear distinction to the empty vector control. Due to the CaMV35S, known as a strong constitutive promoter (Stöger et al. 2005), this low outcome is unexpected. The promoter is able to express a scFv antibody in tobacco leaves up to 6.8 % of TSP (Fiedler et al. 1997).

Although no matches were found in MEROPS database, specific sequences at protein level of VacZP2 could be consensus sequences for proteases. Proteolytic degradation of heterologous proteins is the major factor for low accumulation (Desai et al. 2010). A stabilization of the VacZP2 by fusion or co-expression of stabilizing partners/agents or gene sequence optimization to avoid protease consensus sequences are two measures (Desai et al. 2010; Benchabane et al. 2008). Also, a change of the subcellular localization, already suggested for VacZP2 expression in carrot calli, could be an approach to increase vaccine accumulation. A good example for this is the expression of the human epidermal growth factor (hEGF) via CaMV35S in tobacco leaves. Though, mRNA transcript level was high, the expression in the cytosol was with 0.001 % of TSP low (Higo et al. 1993). A localization to the apoplast increases the accumulation to up to 0.11 % of TSP (Wirth et al. 2004). Expression platform and expression vector have to be appropriate to each other (Gecchele et al. 2015) and our p35S-VacZP2 seems not to fit to tobacco leaf expression under these conditions.

Due to the low number of 12 gained positively transformed events, it is not excluded, that none of these events exhibits a good insertion locus. Mikschofsky et al. 2009b gained only one line exhibiting the heterologous protein CTB::VP60, although 19 DNA-positive transgenic tobacco events were created. It is always better to generate a great number of transgenic events to get a high expressing events (Boothe et al. 2010).

Tobacco seeds

VacZP2 and VacZP3-P were accumulated up to 0.25 and 0.03 % of TSP, respectively. In comparison to other seed-specific expressed biopharmaceuticals the accumulation level is quiet low (Boothe et al. 2010). We used the highly potent *arcelin5*-I promoter for seed-specific expression. It was derived from the garden bean (Romero Andreas et al. 1986) and controls the expression of arcelin, a lectin serving for herbivore resistance in the cotyledons (Goossens et al. 1994). Jaeger et al. 2002 showed with this promoter a more than 100-fold higher yield of a scFv nanobody per gramm (36.6 % of TSP) in *Arabidopsis*. Host, transgene and terminator are different to our setting and have major influence of the outcome. However, which of these factors should be adjusted to increase the accumulation level of VacZP2 and VacZP3-P is not clear so far.

Tobacco seeds as host is potent for expressing high levels of heterologous proteins (Floss et al. 2009, 2009; Tiwari et al. 2009; Scheller et al. 2006; Petruccelli et al. 2006; Ramírez et al. 2001; Tackaberry et al. 1999). Besides *Arabidopsis* (Jaeger et al. 2002; Goossens 1999), Tepary bean (Goossens 1999) and pea seeds (Dr. Huckauf, personal communication), we showed, that the legume *arcelin5*-I promoter is also transferable to tobacco. It is not new to transfer a legume promoter to tobacco. The legume phaseolin promoter accumulated high level of up to 1.1 µg monoclonal antibody per mg dry tobacco seeds in the first generation (Sengupta-Gopalan et al. 1985). This validates a general transfer of legume-specific promoters to tobacco seeds as high potential (Hernández-Velázquez et al. 2015). But it is possible, that the *arcelin5*-I promoter performs not so well in tobacco than in *Arabidopsis*, similar to the phaseolin promoter (Hernández-Velázquez et al. 2015; Morandini et al. 2011). If a morphological difference between *Arabidopsis* and tobacco seed, e.g. the structure of the endosperm, could affect the capacity to store less heterologous protein or transcription and protein synthesis machinery of *Arabidopsis* is more compatible with the regulatory sequences of the vector is not clear (Morandini et al. 2011). A change of host for VacZP2 and VacZP3-P expression could be an option to improvement promoter activity and RNA/protein stability, respectively.

The transgenes of VacZP2 and VacZP3-P could also influence the performance of *arcelin5*-I promoter. Boothe et al. 2010 shows in *Arabidopsis* seeds that accumulation of the bovine chymosin is equal under the control of phaseolin and arcelin promoter, but arcelin promoter is much less efficient than phaseolin when expressing maize oleosin. In our case, sequences and lengths of the constructs seem to be important. The sequence of VacZP2 is 57 % shorter than VacZP3-P and they differ within the antigen domain. Other domains and regulatory sequences are the same. But this difference leads to an 8-fold difference in accumulation level (VacZP2 > VacZP3-P). Sequence truncation of the 65 kDa isoform of human glutamic acid decarboxylase (hGAD65) resulted in an altering accumulation level in tobacco (Merlin et al. 2016). Here a positive correlation between improved solubility and protein stability in the transient expression system was assumed. Whether stability or expression strength is influenced by the transgenes of VacZP2 and VacZP3-P is not clear.

(Jaeger et al. 2002) used an arcelin terminator. This 5’ regulatory element could be more suitable for seed specific expression than the CaMV 35S terminator we used. It is known, that expression under the CaMV promoter and terminator is less effective in seeds (Nausch et al. 2012a). It is possible, that the 35S terminator lowers the high expression rate of the *arcelin5*-I promoter in the tobacco seeds. A change to the 3’ regulatory sequences of the arcelin gene (Goossens et al. 1999) could increase accumulation level of VacZP2 and VacZP3-P by providing a more appropriate combination to the promoter (Nagaya et al. 2010).

Since tobacco seeds offer a protective environment for heterologous proteins (Fiedler and Conrad 1995), the CTB concentration measured by ELISA did not significantly decrease during the experiment of 18 and 84 weeks for VacZP2 and VacZP3-P, respectively. The stability of VacZP2 was only tested for 18 weeks. It is probable that it is similarly stable like VacZP3-P. Because neither detection of VacZP2 nor VacZP3-P via the anti-CTB Western blot analysis was achieved, it is not clear, if the whole CTB-VacZP fusion protein was stable. In comparison to the stability of scFV antibodies for 52 and 72 weeks in

transgenic tobacco seeds (Ramírez et al. 2001; Fiedler and Conrad 1995), VacZP2 is equally stable. VacZP3-P has to be tested again after 84 weeks of storage to compare it to VacZP3-P.

Pea seeds

VacZP3-P was also expressed in pea seeds. It was accumulated in pea seeds at top event 16 up to 0.00008 % of TSP. The accumulation level is very low in comparison to other pea seed-specific expression studies. For example, Saalbach et al. 2001 accumulated a scFv antibody via USP promoter to up to 2 % of TSP and Zimmermann et al. 2009 obtained approximately up to 0.4 % of TSP. In comparison to the expression of the CTB::VP60 fusion protein, the VacZP3-P accumulation is nearly 2,500-times lower (Dr. Huckauf, personal communication). Thus, they use exactly the same expression vector the only difference is the protein itself. So, the construct design and protein determine the stability and in case of the ZP vaccines it seems not very stable or no event with a good integration locus was obtained. The number of events should be increased.

Comparison between carrot and tobacco leaf

In carrot calli the expression under the control of the CaMV35S of VacZP2 was 16-fold higher than in tobacco leafs regarding to the dry mass. The reasons for this difference could be the plant species. They harbor different kinds of proteases and enzymes due to their different physiological setup as totipotent cells and photosynthetic tissue, respectively. Artificial cultivation conditions during *in-vitro* cultivation could cause somaclonal variation and stress leading to transgene inactivation (Mikschofsky et al. 2009c). These stress factors are different from those affecting the vegetative tobacco leave tissue (Gaspar et al. 2002). The accumulation level strongly depends on the transgene itself and the combination of the regulatory elements 5' and 3' (Nagaya et al. 2010). E.g. the expression of VP60 in other plant species than carrot (0.084 µgVP60/mgTSP) exhibited an increase in tobacco (0.5 µgVP60/mgTSP) and potato leaves (2.5 µgVP60/mgTSP) (Mikschofsky 2006). To take the advantages of suspension culture, another cell suspension system like tobacco BY2 or rice or/and the change of the promoter might show higher yields. Shin et al. 2003 has shown that the change of the expression system from tobacco cell suspension with a constitutive promoter to rice cell suspension with an inducible promoter increased the accumulation of human granulocyte-macrophage colony stimulating factor (hGM-CSF).

Comparison pea and tobacco seeds

The extreme difference (583-times) of VacZP3-P accumulation in tobacco and pea seeds under the control of the arcelin promoter could be reasoned by the host. Both plant species own proteases with individual cleavage sites (MEROPS) and it is well-known, that host-own factors determine the levels of accumulation in seeds (Hernández et al. 2013). E.g. phaseolin undergoes no major proteolytic processing in the garden bean, but 70 % is cleaved in transgenic tobacco seeds to smaller peptides (Higgins et al. 1988). This effect of species-specific protein processing/stability and reaction to the expression cassette could also be the reason why Morandini et al. 2011 detected 16- and 21-times less heterologous chimeric GAD67/65 protein driven by an phaseolin promoter in tobacco and petunia seeds than in *Arabidopsis* seeds. These differences in protein processing and stability could also be explained by morphological differences. Pea store most of their

proteins in the cotyledons (Beevers 1968), whereas tobacco stores their proteins in the endosperm (Takaiwa et al. 1995).

Pea (Lau and Sun 2009) and tobacco seeds (Frega et al. 1991) are equal in regard of protein content (approx. 25 % of TSP), but different in yield of seeds per hectare. 2.7 – 3.7 tons pea seeds was harvested in Germany per hectare field and even more in greenhouse is possible (5 tons per hectare (Mikschofsky and Broer 2012)). Tobacco yields 1.1 tons per hectare seeds (Giannelos et al. 2002), which is up to 30 % less. Because of this pea would be the ideal expression platform for VacZP3-P expression. But whether the protein content (Stöger et al. 2002) nor yield per hectare should be the major factor to choose the expression system. Pea is not practical yet, because of low accumulation level. For 1 mg VacZP3-P at least 100 kg pea seeds are necessary. The much better accumulation of VacZP3-P in tobacco seeds overcomes the lower biomass per hectare disadvantage. In the best case 4.2 and 33 g tobacco seeds are sufficient to yield 1 mg VacZP2 and VacZP3-P vaccine, respectively.

Comparison between seed-based and constitutive expression

In comparison to leaf-based expression under the control of the CaMV35S promoter, seed-specific expression of VacZP2 in tobacco seeds was increased up to 2600-times. The improvement to carrot cells was a little less, but with 450-fold still very high. Seed-specific expression could lead to higher accumulation level, than leaf-based expression (Jaeger et al., 2002; Nausch et al., 2012a). The plant organ could have a big influence on protein stability. Petruccelli et al. 2006 detected a proteolytic cleavage of the γ chain of a monoclonal antibody in tobacco leaves, but not in seeds. Due to the desiccant nature of the mature seed and the function as storage organ, proteolytic activity is decreased (Müntz 1998; Stöger et al. 2005), so that recombinant proteins can be stored at room temperature for several months without significant loss (Boothe et al. 2010; Benchabane et al. 2008; Stöger et al. 2005). The pea vicilin storage protein was constitutively expressed in leaf and seed of tobacco. Despite of similar RNA levels the accumulation was 50 – 100-times higher in seeds than in leave tissue (Kermode 2006). Besides the plant organ, the discrepancy of VacZP2 expression between tobacco leaf and seed could be the promoter. Jaeger et al. 2002 increased the expression of the murine scFv G4 from 1 % of TSP in *Arabidopsis* seeds to 12.5 % of TSP by using the *arcelin5*-I instead of the CaMV 35S promoter. The choice of an appropriate promotor to drive protein expression can influence protein level significantly (Stöger et al. 2002).

Transient expression

The transient expression in *N. benthamiana* is known as high production platform for recombinant proteins. Marillonnet et al. 2004 accumulated to up to 5 g_{GFP}/kg_{FW} in *Nicotiana benthamina* after transient expression. For VacZP2 we achieved 0.11 g_{VacZP2}/kg_{FW}. In comparison to Marillonnet et al. 2005 with the same method this is quite low. Nevertheless, our result is in the range of other MagnICON expressed antigens like the isoform of human glutamic acid decarboxylase (GAD65; 0.23 g_{GAD}/kg_{FW}) and human epithelial mucin fused to heat-labile enterotoxin (LTB-MUC1; 0.09 g_{LTB}/kg_{FW}), respectively (Merlin et al. 2016; Pinkhasov et al. 2011). Accumulation of VacZP3-P (0.011 $g_{VacZP3\text{-}P}/kg_{FW}$) was even 10-fold lower. To the best of my knowledge, no data is published such a low level of MagnICON. Other studies expressed a high amount of recombinant protein in

N. benthamiana with the same system (Ponndorf et al. 2016; Nausch and Broer 2016b; Nausch et al. 2012a). In comparison to them it seems, that VacZP2 and VacZP3-P are unstable or have a low expression rate. The major factor for this is the sequence, as well as at nucleotide and amino acid level. The sequence could be responsible for reduction in translation rate, because of inefficient codon optimization (Nausch and Broer 2016b). Furthermore, the sequence could exhibit protease sites. Since the magnitude of 10-fold difference in accumulation level between both ZP vaccines is the same in transient tobacco leaves and transgenic tobacco seeds, the hypothesis is supported. VacZP2 could have less negative impact on the hosts expression machinery and/or exhibit higher resistance to proteases than VacZP3-P, leading to higher accumulation (Michaud et al. 1998).

The content of VacZP2 and VacZP3-P in the freeze-dried and pulverized leaf material not decreases significantly for at least 35 and 21 weeks, respectively. The content stability of both ZP vaccines is similar to the transplastomic expression of CTB-VP1 (a polio antigen; Chan et al. 2016) and CTB-ESAT6 (a tuberculosis antigen; Lakshmi et al. 2013). Both were stable for up to 32 and 24 weeks at room temperature after freeze-drying, respectively.

Seed versus transient expression

Seed-specific expression in tobacco of VacZP2 and VacZP3-P under the control of the *arcelin-5-I* promoter showed the highest accumulation level of all tested stably transformed approaches. With 0.25 and 0.03 % of TSP, they show a typical accumulation level for biopharmaceuticals expressed in plants, ranging between 0.1 – 0.01 % of TSP (Doran 2006), respectively. But expression in the transient system increases the amount of VacZP2 and VacZP3-P per TSP to up to 16 and 12-fold. Because of the viral replicon and the resulting higher expression rate, the transient expression is able to deliver high protein yields in a short period of time (Xu et al. 2012), which is an advantage over stable transformation strategies. It was presumed that the sensitivity of the VacZP vaccines limit a higher accumulation in tobacco seeds, the enourmous expression rate of the transient expression system might compensate a protein degradation in long-term (Nausch et al. 2012a). Expression of the Interleukin-6 via the same transformation vector (pICH29912) we used, exhibited up to 64-times higher accumulation than in tobacco seeds (Nausch et al. 2012a). Vaquero et al. 2002 expressed more of T84.66/GS8 diabody in a transient expression approach than with stable transformed tobacco plants.

In some cases stable transformed plants have even higher yield of recombinant protein than transient expression (Merlin et al. 2016; Gecchele et al. 2015), in addition to that, these systems have advantages in product costs and scalability (Xu et al. 2012). Especially, when taking into account that the proportion of recombinant protein can be increased via breeding (Nausch et al. 2012b; Hühns et al. 2008; Jaeger et al. 2002).

The seed-based expression of the VacZP vaccines has a big advantage over leaf-based transient expression, when it comes to storage of unprocessed transgenic plant material. The desiccated nature of mature seeds, together with the low abundance of active proteases in seed tissues during dormancy, prevent extensive proteolysis and promote long-term stability of proteins in planta (Stöger et al. 2000; Fiedler and Conrad 1995). This allows a complete separation of cultivation and processing/purification process without additional conservation (Boothe et al. 2010). Seeds have a simpler protein composition

than vegetative tissue, which could result in fewer purification steps and lower costs (Twyman et al. 2005; Stöger et al. 2002). Leaf tissue have to be conserved or processed immediately after harvest (Xu et al. 2012). Transient expressed VacZP2 and VacZP3-P in leaf material was freeze-dried and stabilized the protein content for at least 21 weeks. This conservation method is an additional cost point in the downstream process and the stability of VacZP3-P content was much lower than in seeds. The low nicotine present in the tobacco seeds allows oral application without expensive purification (Rossi et al. 2003) in contrast to Agrobacterium-contaminated and highly toxic tobacco leaves in the transient expression system (Rosales-Mendoza et al. 2017).

The advantage of the transient expression system MagnICON against the stable seed-based system is the possibility to shortly react to sudden demand of recombinant protein. But production capacity is limited by the technical process of plant transformation. Due to the publication from Hamorsky et al. 2013 we assume that Kentucky Bioprocessing, LLC, a large-scale transformation factory, would be able to produce around 950 g VacZP2 and 77 g VacZP3-P in 12 weeks. The costs and efforts are higher than producing this amount of recombinant vaccine in the tobacco seed-based system. Here only 3.6 (VacZP2) and 2.4 hectar (VacZP3-P) would be necessary, presuming a tobacco seed yield of 1.1 ton seed per hectar (Hernández-Velázquez et al. 2015; Giannelos et al. 2002). Provided that a grow on open field is approved, seed-based production of both VacZP vaccines become an attractive alternative to transient expression, when short reaction time is not mandatory.

Due to the high alkaloid content in the *Nicotiana* leaves (200 mg/kg_{FW}) (Rossi et al. 2013), the ZP vaccines generated via transient expression must be purified for administration. It is not suitable for oral vaccination (Daniell et al. 2009). In contrast to this, tobacco seeds contain much less (2 µg/kg_{FW}) nicotine (Rossi et al. 2013) and can be used for oral vaccination (Rossi et al. 2014), which could save money in the downstream processing due to a simpler process (Nausch et al. 2012b; Ramessar et al. 2008). If a purification is desired, the purification process could be simplified, because of a lower profile of the seed protein (Lau and Sun 2009; Jaeger et al. 2002).

Since the MagnICON platform offers the opportunity of short available biopharmaceuticals (Gleba et al. 2005), we decided to produce VacZP2 and VacZP3 P in *Nicotiana benthamiana* to rapidly gain enough vaccine material for animal testing via parenteral injection. This process is more cost intensive than seed-based production, due to high effort of infiltration and conservation by freeze-drying. Building up a seed-based VacZP expression takes around 6 months longer in the beginning, but once established it costs only a few amounts of money. If the factors of up-scaling and oral vaccination become more important, than the use of the seed-based expression system should be considered.

Plant versus *E. coli*, yeast, CHO cells

The expression of VacZP vaccines were performed in plants, because of special features in comparison to other pro- and eukaryotic expression systems. Plants have similar post-translational modifications like animals and humans with minor difference in protein glycosylation (Fischer and Emans 2000). This glycosylation pattern could contribute to a native epitope of an VacZP antibody, which seems to be critical for sterilizing (Hardy et

al. 2003) and due to plant specific α1,3 fucose and/or β1,2 xylose may also increase immunogenicity of the vaccines (Matoba 2015; Bosch and Schots 2010; Saint-Jore-Dupas et al. 2007). In contrast, *E. coli* has no glycosylation and yeast very different pattern in comparison to mammals. The mannan-type N-glycans of yeast are more immunogenic than plants and can lead to unwanted reactions after administration (Gomord and Faye 2004).

Purification from plant cells is much simpler and saver, than from CHO cells, because of not hosting animal and human pathogens (e.g. HIV, hepatitis viruses) (Doran 2000). This lack of screening has a favorable effect on production cost. These cost are in general lower than CHO cell expression (Fischer and Emans 2000). Because of the independence from fermenters transgenic plants are easy to scale up and to propagate, which is a big advantage over microbial and mammalian cell cultures (Fischer and Emans 2000).

Both VacZP proteins exhibited instability in *N. benthamiana in vivo* and *in vitro*. In case of VacZP3-B this was also observed during bacterial expression. After extraction VacZP3-B was stable but degradation started when plant protein extract was added. To avoid degradation of plant-derived VacZPs in and ex planta the targeting to an alternative intracellular compartement (e.g. plastids, vacuole) (Santos et al. 2016) or fusion to an stabilizing agent (Lau and Sun 2009) should be taken into account.

5.2 VacZP2

The highest yield of around 1 µg per mg dry weight for VacZP2 was observed after transient expression in *N. benthamiana*. Though the amounts of our ZP vaccines produced in plants are low compared to other plant made recombinant proteins, to gain 100 µg ΔpZP2 only 2 g of fresh plant material is sufficient. To gain the same amount from the natural source, up to 227 and 104 pig ovaries are necessary, respectively (Hasegawa et al. 1991; Hedrick and Wardrip 1987; Dr. K. M. Frank, SCC, Billings, MT, personal communication). A dose of PZP, containing porcine ZP2, ZP3 and ZP4, is 65 – 100 µg for a wild horse.

It is still questionable, whether sufficient amounts of pig ovaries are available for immunization of great numbers of small mammals like dogs, cats or rodents. In addition, the plant made vaccine is free from contamination with other proteins present in the ovary that lead to inflammation in many recipients (Gupta et al. 2014).

Infiltration of pICH-VacZP2 with the MagnICON vector elicited strong phenotypical changes on *N. benthamiana* leaves. This observation is common (Huy and Kim 2017; Nausch and Broer 2016b; Hamorsky et al. 2015; Pinkhasov et al. 2011; Huang et al. 2006), and not referable to the transgenes. But leaves infiltrated with a ZP construct were more healthy compared to the empty vector control (Nausch and Broer 2016b). It still remains unclear, whether the ZP vaccines are harmful to the plants by themselves or viral replication exhaust plant translation machinery. However, no phenotypical saliences in plant growth were observed in the stable transformed plants, which most likely make a harmful effect of VacZP2.

The quality (integrity) of VacZP2 protein via MagnICON expression was analyzed in Western blot analysis. Antibodies binding at the N-terminal CTB and the C-terminal his-tag demonstrate the presence of the complete fusion protein, respectively. The fusion protein was purified via Ni-NTA purification. In the elution fraction besides VacZP2 CTB

monomers were always detectable. Gentle and protease protected isolation conditions had no effect on the occurrence of CTB monomers in the elution fraction. A co-purification of free CTB via Ni-NTA columns is probable (Dertzbaugh and Cox 1998). But it is also possible, that CTB monomers assemble with VacZP2 monomers to pentamers and are co-purified via his-tag.

Directly after crude protein isolation from lyophilized *N. benthamiana* leaves, VacZP2 is degraded. Responsible for this might be plant proteases. This is supported by the fact, that low temperature or protease inhibitors retard this *in vitro* reaction. It can be excluded that oligomerization of VacZP2 is the reason for the fading of the monomer signal, because of a non-increasing signal for oligomers and stopped signal fading after purification.

VacZP2 is able to form pentamers via the CTB domain. This was verified by mono-sialoganglioside GM1 binding assays (Dakterzada et al. 2012; Merritt et al. 1994; Hardy et al. 1988). Our results indicate that the majority of measured CTB is pentameric (unpublished data), what is conform with the auto-assembling ability of CTB (Yasuda et al. 1998). Due to other CTB fusion protein studies (Huy and Kim 2017; Nochi et al. 2007), it could be assumed that the oligomerization ability of our CTB-ZP fusion protein is also necessary for a possible mucosal delivery.

The detection of VacZP2 in the lectine blot and a slightly heavier monomer protein (approx. 35 kDa) than calculated (32.2 kDa) proofs an occupation with *N*-glycans. Due to four *N*-glycosylation sites in the VacZP2 sequence a macroheterogenic in *N*-glycosylation (Jones et al. 2005) was expected, which would lead to VacZP2 variants of alternating weight between 35 – 44 kDa. This could explain the occurrence of a second slightly heavier band (approx. 41 kDa) of the VacZP2 monomer. A variation in glycosylation of heterologous glycoproteins in plant expression systems is very common (Gomord and Faye 2004; Bardor et al. 2003b), so that two VacZP2 monomer variants are not unusual. This inconsistency of site occupation was previously observed in tobacco (Hamorsky et al. 2013) and rice (Kajiura et al. 2013; Yuki et al. 2013), which resulted in a non- and once glycosylated CTB with a difference in mass between 1.5 and 3 kDa (Mikschofsky et al. 2009a; Matoba et al. 2009; Mishra et al. 2006). Plant-based CTB expression in other studies observed a macroheterogeneously glycosylated CTB at Asn4 (Hamorsky et al. 2013; Kajiura et al. 2013; Yuki et al. 2013; Mikschofsky et al. 2009a; Nochi et al. 2007), what could explain the lighter (12 and 15 kDa) bands. The third band (18 kDa) could originate from microheterogenous occupation (Jones et al. 2005) of Asn4 or glycosylation at Asn93, a second potential Asn-X-Thr/Ser sites (Kornfeld and Kornfeld 1985). But occupation of Asn93 seems less probable (Matoba 2015) due to subsequent proline after the sequon (Gavel and Heijne 1990; Bause 1983). With our results, no exact statement is possible, which one of the four Asn-X-Thr/Ser sites (Kornfeld and Kornfeld 1985) is occupied. Two are situated in the CTB domain and two in the $_{\Delta}$pZP2 domain. However, due to occurring free glycosylated CTB molecules between 12 and 18 kDa when VacZP2 is expressed, a glycosylation of the CTB domain within the VacZP2 fusion protein could be assumed. Furthermore, the occupation of the $_{\Delta}$pZP2 domain could be assumed, because the plant-derived $_{\Delta}$pZP2$_{only}$P is also glycosylated and a likewise occupation of the *N*-glycosylation sites is possible.

Free CTB molecules between 12 and 18 kDa were detected, despite of protective protease inhibitor mix and cooled and gentle isolation of VacZP2. That indicates a possible *in vivo* instability at protein or RNA level (Stöger et al. 2002). In regard to protein instability, free CTB molecules could represent C-terminally truncated VacZP2 molecules, like the CTB-$MPR_{649-684}$ from Matoba et al. 2009. Similar to this study, the rest of our fusion protein (antigen, TT, histidine tag and SEKDEL) could not be detected via anti-histidine antibodies. Maybe it was degraded by exopeptidases beginning from the C-terminus of VacZP2 and CTB is a leftover, because it is known for their stability in harsh environments (Zhang et al. 1995). The phenomenon of free CTB molecules was observed by other studies in transplastomic tobacco (Chan et al. 2016; Lakshmi et al. 2013) and *N. benthamiana* in an ER-targeted approach (Tien et al. 2017; Kim et al. 2016; Matoba et al. 2009; Huckauf J., unpublished data). Another possible explanation could be a split in the Glycin-Proline-Glycin-Proline (GPGP) hinge region between CTB and antigen by an uncharacterized plant endopeptidase (Matoba et al. 2009). But this observation is inconsistent with other CTB-based vaccines using GPGP linker expressed in transplastomic tobacco (Su et al. 2015a; Su et al. 2015b; Kwon et al. 2013b; Boyhan and Daniell 2011; Davoodi-Semiromi et al. 2010; Verma et al. 2010; Ruhlman et al. 2007). In this study it was not possible to determine a responsible *N. benthamiana* specific protease via MEROPS protease database, which is cutting in the region of the GPGP linker and bring an explanation for CTB monomer.

But free CTB molecules could also be a production of transcript instability or an unexpected alternative transcription. If there is a separate transcript leading to free CTB monomers a mRNA fitting to detected proteins would be detectable. Anti-CTB Northern blot analysis detected RNA (approx. 483 nt) longer than CTB domain + GPGP linker (390 nt) and much shorter than the smallest viral mRNA coding for the VacZP2 fusion protein (1178 nt). One possibility could be an unwanted stop during *vacZP2* transcription from the gen inside the nucleus or the viral replicon in the cytosol. But no transcription stop signal within the sequence of *vacZP2* was found to describe such small RNA fragments. The consequences of free CTB in the plant protein extract are discussed in the last chapter.

VacZP2 was parenterally delivered in female mice and hamsters and show an immune reaction. In mice immunization show an anti-VacZP2 immune reaction already after priming, which is a typical course (Janeway and Murphy 2012; 8th ed.; p. 24). The titer increases with additional booster, which is an effect of the immunological memory (Prisco and Berardinis 2012; Sallusto et al. 2010). Interestingly, the anti-$_{\Delta}$pZP2$_{only}$P titer significantly reduces after second boost, which could be a sign of too short boost interval resulting in terminally differentiated memory T cells with decreased capacity to proliferate (Sallusto et al. 2010). The reduction after the second boost in antibody titer in the same mice strain and same time interval was also observed by Gupta et al. 2013. That booster injections doesn't lead to an increase of antibody titer is not specific for recombinant ZP vaccines in mice (Mahi-Brown et al. 1992; Keenan 1991; Wood 1981). Especially, the vaccination of hamster with PZP did not increase the antibody titer after third boost (Hasegawa et al. 1992). This is consistent to our results in hamster with VacZP2. A optimization of the priming-booster regime could intensify the immune reaction (Castiglione et al. 2012) and longer periods of time between booster injections could elicit higher amplification (Sacco et al. 1983). But it could also be possible that a limit was reached like in many other ZP studies (Kitchener et al. 2009b; Kitchener et al.

2002; Hasegawa et al. 1992; Hasegawa et al. 1991; Keenan 1991; Sacco et al. 1981; Wood 1981).

Interestingly we observed an immune reaction without adjuvant in mice. The level of antibodies against the antigen domain $_{\Delta}$pZP2 is actually equal to vaccination with adjuvant after the second boost. Immunization studies of PZP without adjuvant resulted in a poor (Mahi-Brown 1985) or no (Bhatnagar et al. 1989) immune reaction and it must be given with an adjuvant (Kirkpatrick et al. 2011). This could be an indicator for good intrinsic immunogenicity of VacZP2 by a sophisticated vaccine design (De Groot, Anne S. et al. 2010), maybe triggered by the fused adjuvants CTB (Chan et al. 2016) and TT (Fraser et al. 2014; Panina-Bordignon et al. 1989), the plant specific glycosylation pattern (Bosch and Schots 2010; Matoba et al. 2009; Bardor et al. 2003a; Kurosaka et al. 1991) or plant-derived adjuvant compounds (Rosales-Mendoza and Salazar-González 2014). Besides this, the integration of the di-lysine linker, a cleavage site of the cathepsin B protease (Sarobe et al. 1993; Takahashi et al. 1988), could improve the antigen processing of VacZP2. The feature of no need for an adjuvant was shown before with other CTB fusion proteins (Kwon et al. 2013a; Ruhlman et al. 2007) and may dispense the necessity of an extra formulation step of the plant material to sterilize animals.

Antibodies against the $_{\Delta}$pZP2 domain, proven by anti-$_{\Delta}$pZP2$_{only}$P ELISA, revealedthat the design of the VacZP2 fusion protein enables an appropriate antigen presentation in the animal. This is a requirement of the formation of sterilizing antibodies. How much the ratio of $_{\Delta}$pZP2 antibody to antibodies of $_{\Delta}$pZP2 fusion partners is could not be determined. A comparison by serum dilution is invalid because no correction factor or unifying standard was used in both assays. How much antibodies binding to a immobilized antigen, which is measurable by the extinction, depends on the affinity (Heinrich et al. 2010). This affinity, composed of the dissociation constant and the kinetics (Heinrich et al. 2010), was not determined as correction factor.

The same counts for the comparison between mice and hamster. It is not accurate to say in which animal VacZP2 is more immunogenic, because the quality of the immune response resulting in a reduced fertility is relevant (Jones et al. 1992). But a differing immunogenicity of VacZP2 could be assumed, because of different genetic background and the connected immune system (Dunbar et al. 1994).

A subsequent mating experiment to show how contraceptive VacZP2 in FvB mice is, failed due to highly aggressive female mice. They have bitten males and killed their offspring, although this they were acclimated to their housings (unpublished data). A repeat with more calm mice, maybe another mice strain, could answer the question of spaying efficacy of VacZP2 in mice.

VacZP2 show an impact on hamster fertility. This could originate from an included epitope (5H4) with known contraceptive effect *in vitro* (Hasegawa et al. 2002; Hasegawa et al. 2000; Shigeta et al. 2000; Hasegawa et al. 1995) and *in vivo* (Miller et al. 2000). The effect on contraception is not significant and lower than observed with native, porcine $_{\Delta}$ZP2 (Hasegawa et al. 1992; in this paper designated as ZP4). The quality of the VacZP2 antibodies should be approved by IVF (in vitro fertilization) assay. This would help to find out whether the antibody titer is under the threshold of blocking sperm-egg contact or the fertilization-relevant epitopes on the hamster’s ZP are not occupied. First, if the titer

is to low, stronger adjuvants (Bagavant et al. 1994) like complete Freund's adjuvant (CFA) used by Hasegawa et al. 1992 during hamster immunization or a more optimal dose could be a solution (Mahi-Brown et al. 1992). Second, if the relevant ZP region is not covered by VacZP2 antibodies to block sperm from fertilizing, the sequence design of VacZP2 should be optimized to support an appropriate antigen processing inside hamster's leukocytes, meaning making 5H4 and other unknown epitopes available. This measures includes the prevention of interfering plantal PTMs, like epitope covering glycan residues (Matoba 2015; Boes et al. 2015), and positive sequence adjustments, like the introduction of spacers between the ZP epitopes or fusion of other promiscuous epitopes (De Groot, Anne S. et al. 2010).

Vaccination with VacZP2 + Polygen seems to have a transient effect on fertility. This is supported by histological analysis of hamster ovaries (unpublished data). The ovar exhibited no unnormal atresia or inflammation (Dr. J. Schön, FBN Dummerstorf, personal communication), having a healthy pool of follicles able to develop fertile eggs. In contrast, the vaccination of hamster with PZP + CFA (Hasegawa et al. 1992) and recombinant Hamster ZP (Koyama et al. 2005) causing huge ovary degeneration in the follicle pool, leading to permanent sterility by cytotoxic immune response. It is possible, that VacZP2 cause no degeneration due to a humoral immune response, where only antibodies against the egg are generated. Which of the immune reaction is triggered, is mainly controlled by adjuvants (Vogel 2000) and it is possible, that CTB (Baldauf et al. 2015) and TT (Panina-Bordignon et al. 1989) are also involved in eliciting a humoral response. Bonnet monkey ZP3 caused with CFA profound follicular atrophy in marmosets, but not with SPLPS (Upadhyay 1989). For VacZP2 + Polygen subsequent analysis about the type of immune response (Th1 or Th2) should be conducted. Another possibility, why no follicles were negatively affected, could be that anti-VacZP2 antibodies are not affine to the ZP of early stage follicles. In this case they are not able to degenerate the whole follicle pool (Rankin et al. 2001). This is supported by the fact, that $_{\Delta}$pZP2 epitopes are presented not until late stages of follicle development (secondary and tertiary follicle) in hamster (Hasegawa et al. 1992).

VacZP2 is still in a concept phase and thus not economical. One reason for this is the low level of contraception. The high immunogenic effect of VacZP2 detected in hamster should lead ideally to a block of fertilization at least for 50 % of tested animals and subsequently eliciting a long-lasting protection. Since first was not complied in our experiment, second is obsolete. The next step should be the improvement of the immune response quality by dose, formulation and prime-booster regime studies, to get equal to native $_{\Delta}$pZP2 in hamster. Not before the potential of contraception of VacZP2 in hamster has been exhausted, it is difficult to conclude how much VacZP2 should be produced to sterilize a specific number of animals. So, besides the immunogenic efficacy the production platform defines the costs. The plant production system determines the speed and effectivity of production due to yield per biomass, time of production cycles, storability and costs of cultivation and purification. We only tested the transient expression system to produce mid amounts of VacZP2. The seed system could become attractive, when accumulation level is increased. The advantages are a long storability without special conservation methods, great scalability and proper for oral application. But and economic comparison between transient and seed-based expression system could not be done in this work. But expression of VacZP2 in carrot cells and tobacco leaves

under the control of the CaMV35S can be designated as unpractical in this setting. To gain 1 mg of VacZP2 at least 1.9 kg carrot callus is required. To gain 1 mg vaccine 30 kg tobacco leaf material has to be harvested.

5.3 VacZP3-P

The production of VacZP3-P was best implemented with transient expression system. Although the yield of VacZP3-P is low compared to other recombinant plant made biopharmaceuticals, only 18 g of fresh *N. benthamiana* for 100 µg ZP3 are sufficient. We assume, that up to 104 pig ovaries are necessary to receive the same amount (Hasegawa et al. 1991; Hedrick and Wardrip 1987; Dr. K. M. Frank, SCC, Billings, MT, personal communication with). It is obvious, that recombinant ZP vaccines could overcome limited availability of pig ovaries to immunize a large number of mammals (Gupta 1997). The plant made VacZP3-P is free from contamination with other proteins leading to inflammations in the ovary (reviewed in Gupta et al. 2014).

Similar to the transient expression of VacZP2, the expression of VacZP3-P causes massive leave withering. But leaves infiltrated with empty vector showed even higher damage, too. This relieves VacZP3-P from being harmful to the plant. This is supported by the expression in pea and tobacco via pARC-VacZP3-P, where no special phenotype was exhibited. Exception was tobacco event 144 with a reduced seed production and highest VacZP3-P accumulation level. Our phenotype could also be addressed to somaclonal variations (Kaeppler et al. 2000). Further studies show that only 10 % of the offspring still have this phenotype. The other 90 % show no phenotype but have similar accumulation levels (Jana Huckauf, personal communication).

The integration of VacZP3-P was verified by Western analysis targeting the N-terminal CTB and the C-terminal his-tag. But similar to VacZP2 free CTB molecules occurred as well as in the crude protein extract and the elution fraction. So, there were the same problems to quantify VacZP3-P via anti-CTB ELISA and isolating VacZP3 from free CTB molecules.

VacZP3-P is also sensitive to plant proteases after isolation and degrades without protection by cooling or protease inhibitors. The reason for degradation should lay within plant protein extract. This is supported by the degradation of purified bacterial VacZP3-B by crude protein extract from tobacco.

VacZP3-P pentamer formation was also proven by GM1 binding assay like VacZP2-P. That means VacZP3-P is due to the fusion of CTB able to bind to many cell types (Sánchez and Holmgren 2008), even gut epithelia cells. CTB enables an oral route through the M-cells (Nochi et al. 2007). Thus, amount detected in anti-CTB and GM1 binding assay are not significantly different, a pentamer formation of the majority of CTB molecules could be assumed.

VacZP3-P is *N*-glycosylated. The difference in size of 17 kDa between the unglycosylated, bacterial VacZP3-B to the plantal VacZP3-P could be explained by the occupation of N-glycans. Due to free, glycosylated CTB molecules, an occupation within the CTB domain like VacZP2 could be assumed. But because of the size other glycosylation sites within the antigen domain should be occupied. Which of the 5 sites offsite from CTB is occupied could only be cleared by mass spectrometrie.

It is interesting, that the phenomenon of free CTB molecules occurs as well as by VacZP3-P and VacZP2 expression. And it is still not clear at which level (RNA/protein) the trigger for CTB formation is situated. Thus, no equal sequence or motif in the N-terminus of both ZP domains were found, it is more likely that the equal coding region or amino acid sequence of CTB and GPGP linker is responsible for causing a transcriptional/translational error and proteolytic degradation, respectively. This hypothesis is supported by other studies using CTB-GPGP combination (see discussion VacZP2).

The *in vitro* degradation of purified VacZP3-B by plantal crude protein extract created no CTB monomers. For plantal CTB formation it means, that this phenomenon supports both in vivo CTB formation by plantal ER proteases before cell breakdown and an unwanted interruption of the transcription/translation to the fusion protein. Both processes happen only in intact plant cells. The absent formation of novel CTB monomers during in vitro degeneration of plantal VacZP3-P would support it. How free CTB influences the immunogenicity is discussed in the last chapter.

The plant-made VacZP3-P elicited a good immune response in BALB/c directly after priming and increased after each booster injection significantly even at low dosages. Before the second boost, higher doses show no significant difference on antibody formation. This is the first time, that such small amounts of 2 µg porcine ZP vaccine were tested in mice and show immune response (Sacco et al. 1981; approx. 3.3 µg). However, after second boost, group with the highest dose (21 µg) exhibit a significantly higher antibody titer against VacZP3-P. The 1.25-fold increased in antibody titer after second boost with 21 µg were realized by a 10- fold higher dose in comparison to 2 µg. If this little difference in antibody titer is critical for sterilizing or could be neglected could only be evaluated by subsequent mating experiment (Kirkpatrick, History of PZP, unpublished data). In our experiment, there was no direct connection between anti-VacZP3-P antibody titer and infertility. But it needs to be taken into account, that the titer against the sterilizing pZP3 domain was not measured and that the anti-VacZP3-P titer may include also antibodies against CTB, TT and his-tag. Due to the VacZP2 titer results, where high titer against VacZP2 also exhibited high titer against the antigenic $_{\Delta}$pZP2 domain, we assume the same for VacZP3-P titer. Some individuals with high VacZP3-P titer became pregnant others didn’t or exhibited a reduced litter size. In some studies there are correlations between antibody titer and impact on fertility (Lloyd et al. 2003; Hardy et al. 2002b; Govind et al. 2002; Sadler et al. 1999; Liu et al. 1989; Millar et al. 1989; East et al. 1985), in others don’t (Clydesdale et al. 2004; Lai 2004; Lloyd et al. 2003; Hardy et al. 2003; Srivastava 2002; Martinez 2000) or the effect is inverse (Lo et al. 2011). This phenomenon could not be limited to a specific type of vaccine or animal species. The qualitive nature of antibodies seems more significant than absolute titer (Hasegawa et al. 2002; Jones et al. 1992; Sacco et al. 1981).

Even though in our approach no connect between titer and fertility was observed, statistically a direct connection between dose and the number of offspring is visible. It could be possible that we increase the contraceptive effect on mice after VacZP3-P immunization by increasing the dose to an optimum. An increase of VacZP3-P dose could enhance the sterilizing effect. The optimal dose for native PZP in mice was determined with 137 µg (Sacco et al. 1981), which is more than 6-times higher. But the optimal dose depends on the vaccine itself and should be determined by further experiments.

The mating experiment showed a tendency of impact on mice fertility by VacZP3-P. This is supported by a reduction of 43 % of offspring in the group receiving 21 µg VacZP3-P (animals failed to conceive are included). This is much less than the reduction of mice litter size of 74 and 90 % Clydesdale et al. 2004 and Gupta et al. 2013 obtain with their recombinant porcine ZP3 vaccines, respectively. In our approach, the reduction of the litter size is not significant to the control group ($p = 0.06$, Tukey-HSD; $p = 0.085$, Bonferroni test), which is a similar observation like Sacco et al. 1981 made with native PZP, which contain pZP3. This group discussed that antibodies against pig zona pellucida maybe doesn't bind to murine fertilization-relevant epitopes, but this is disproved by the recombinant vaccines from Clydesdale and Gupta. A second experiment with VacZP3-P with the same setting reproduced the sterilizing effect to BALB/c mice (Dr. Huckauf, personal communication). Although our approach used the same porcine ZP3 antigen like Clydesdale et al. 2004 and Gupta et al. 2013 many things are different: vaccine design, expression system, mice strain and adjuvant.

VacZP3 uses other fused adjuvants than Gupta et al. 2013 to increase the immunogenicity. Clydesdale et al. 2004 and Gupta et al. 2013 expressed their vaccine in *E. coli* and simian kidney fibroblast cells, respectively, which results in different PTMs (post translational modifications). We used BALB/c mice like Clydesdale et al. Gupta immunized FvB/J mice with the synthetic polymer adjuvant PetGel A. Clydesdale et al. 2004 used Complete Freund's adjuvant (CFA or FCA), the strongest adjuvant, which could cause unwanted inflammations at the infection site (Sacco et al. 1989) or the ovar (Gupta et al. 2013; Frank et al. 2005; Upadhyay 1989). In our experiment the co-polymer adjuvant Polygen was used, a strong adjuvant with proven record (Wernike et al. 2017; Mikschofsky et al. 2009b; Wegelt et al. 2009). These differences make it difficult to determine one factor, which could be responsible for the lower contraceptive effect in our experiment setting. Additionally, Polygen as adjuvant for ZP vaccination was used for the first time. But The much lower efficacy of TT-KK-ZP3 from Gupta et al. 2013 in our setting, other mice and other adjuvant, is exemplary for the high influence of genetic animal background (Bagavant 1997; Lou et al. 1995; Rhim et al. 1992) and adjuvant (Gupta et al. 2011; Upadhyay 1989) a on contraception.

Although we used a similar size per group (Gupta et al. 2013; Hardy et al. 2004; Clydesdale et al. 2004; Sacco et al. 1981), the spaying effect within our testing groups were very variable. Some individuals show normal litter size other only reduced ones. This contributed to a high standard deviation leading to a fail of significance. It is not clear, if an increase of group sizes vaccinated with VacZP3-P would sharpen the statistics. At this point VacZP3-P only affects the fertility of special individuals, although all mice threated with 21 µg vaccines respond homogenous. But this observation occur also in other BALB/c mice ZP immunization studies (Hardy et al. 2008; Hardy et al. 2004; Jackson 1998), though the amount of responders was higher than 50 %. This is the reason existing antibodies decrease fertility or remain uneffective is still unclear. But the individual hormone profiles of the female mice could vary during the experiment and could have an influence on the ZP structure (Barber and Fayrer-Hosken 2000). In further experiments *in vitro* fertilization should be conducted to prove an anti-VacZP3-P antibody binding to the egg. This is the requirement for shielding the sperm receptor on the egg from the sperm contact.

The question whether VacZP3-P is economic at this point, could be answered like for VacZP2. The contraceptive effect on mice and the accumulation level in the transient expression system is yet to low. Both factors have to be increase with further work. It could also be possible that one factor limits the other. If this occurs, a change of expression system or animal species should be considered. It was shown, that PZP is not very potent in mice (Martin et al. 2006; Sacco et al. 1981) and kittens (Gorman et al. 2002; Jewgenow et al. 2000), but effective in marsupials (Kitchener et al. 2009b; Kitchener et al. 2002) and ungulates (Frank et al. 2005). But a final evaluation should be done after optimization at production and application side. The first results are promising to do so.

5.4 Comparison between VacZP2 and VacZP3-P

A final verdict whether VacZP2 or VacZP3-P is more contraceptive and should be favored to spay mammals, is not possible at this point. The accumulation level in plant expression systems clearly supports VacZP2. In the transient expression system, where both vaccine performed best, VacZP2 accumulated over 11-fold higher than VacZP3-P. But in regard to the efficacy in animals a comparison is vague. Both prove their immunogenic potential in mice, but for VacZP3-P immunization we had to use BALB/c mice, a different inbred strain than used with VacZP2. A comparison of the immune reaction of two mice strains with different MHC H2 haplotypes is invalid. It is known that the genetic background influences the immunogenicity (Hardy et al. 2003; Hardy et al. 2002b; Lou et al. 1995a). Because of this barrier, we cannot say whether VacZP2 or VacZP3-P is more immunogenic. We used a different mice strain to test VacZP3-P to avoid a fail of the mice mating experiment like with VacZP2 once again. During the mating experiment with VacZP2 the FvB/NcrL mice showed high aggression and corrupted the results. We decided against a de novo mice experiment with VacZP2, which impeded a direct comparison between VacZP2 and VacZP3-P in regard to contraception. The subsequent VacZP2 test in hamster enabled a direct comparison to the literature of Hasegawa et al. 1992, which is the only *in vivo* mating study using small animals for porcine ZP2 testing. This made an evaluation of the plant-made VacZP2 vaccine in comparison to common ZP vaccines possible. In further work the mating experiment with VacZP2 in BALB/c mice should be conducted, to obtain a better comparability between VacZP2 and VacZP3-P.

In comparison to studies with the recombinant murine ZP2 and ZP3, where a sterilization rate in mice of over 50 % was obtained (Clydesdale et al. 2004; Hardy et al. 2003; Sun 1999; Millar et al. 1989), our vaccines had a low spaying effect. Since ZP2 (Hasegawa et al. 2000) and ZP3 (Gupta et al. 2013; Paterson et al. 1992) are both critical targets to cause a block of sperm contact, it is also possible, that a combination of both plant-made vaccines results in an increased contraceptive effect.

Due to the porcine sequence in VacZP2 and VacZP3-P, it could show a stronger effect in ungulates, than in rodents, like the cross-reactive PZP vaccine (Kirkpatrick et al. 2011; Sacco et al. 1981). Further experiments have to be conducted to test the efficacy in wild animals, like boar or deer, and the mucosal delivery of the antigen via CTB. The accumulation of ZP vaccines in fusion with the mucosal adjuvant CTB-based in edible tobacco seeds is a huge step forward, because it opens an opportunity of novel vaccine delivery (Gupta et al. 2011)

5.5 CTB – A critical point in the method

The detection of free CTB molecules in the Western blot renders the quantification of VacZP2 and VacZP3 via CTB Elisa problematic. Since VacZP2 and VacZP3-P content is measured via CTB, the occurrence of free CTB leads to an overestimation of the active compound. This problem was solved by studies with the same problem by a transient precipitation of the active compound (Matoba et al. 2009) or densitometric approach via anti-CTB Western blot analysis (Lakshmi et al. 2013). Because precipitation of VacZP2 and VacZP3-P was not reversible (data not shown), free CTB molecule quantification in the protein extracts was conducted with the help of densitometric Western blot analysis against commercially available CTB as standard. The determined mass was set in relation to the whole amount of CTB measured via anti-CTB ELISA. The amount of free CTB is similar in both approaches, which exhibit a connection between the expression levels of the fusion proteins.

Free CTB molecules were also present during immunization of VacZP2 and VacZP3-P, respectively. Free, non-antigen linked CTB is weakly immunogenic in contrast to fused CTB (Eriksson et al. 2003). The free CTB molecules in our vaccine should not decrease the immune response and may act as adjuvant improving the immune response via mucosal (Prabakaran et al. 2008; Asahi-Ozaki et al. 2006; Isaka et al. 2001) and parenteral route (Hirabayashi et al. 1990).

5.6 Aims archieved

The six aims (1 – 6) of the concept study were successfully achieved. (1) Two ZP vaccines, VacZP2 and VacZP3-P, in fusion with adjuvants were designed to block the sperm-egg contact. (2) Both were expressed in different stable and transient plant expression systems to cover special features of low costs, high scalability, long storability and high production time flexibility, respectively. (3) In all tested systems the vaccines were detected. The transient system, with the highest yield in a short period of time, was set as production platform. ZP vaccines were (4) purified and (5) characterized to (6) evaluate the immunogenicity and contraceptive efficacy in animals. The results of this study can be used to continue the development of a plant-made ZP vaccine, to achieve theoretically unlimited amounts of recombinant protein connected with low cost. In further studies an increase of the contraceptive effect and the way of delivery should be addressed to be practicle for wild life management (Gupta et al. 2011). The base is set. VacZP2 and VacZP3-P are fused to CTB and expressable in edible plant parts, which allows oral vaccination (Nochi et al. 2007; Haq et al. 1995).

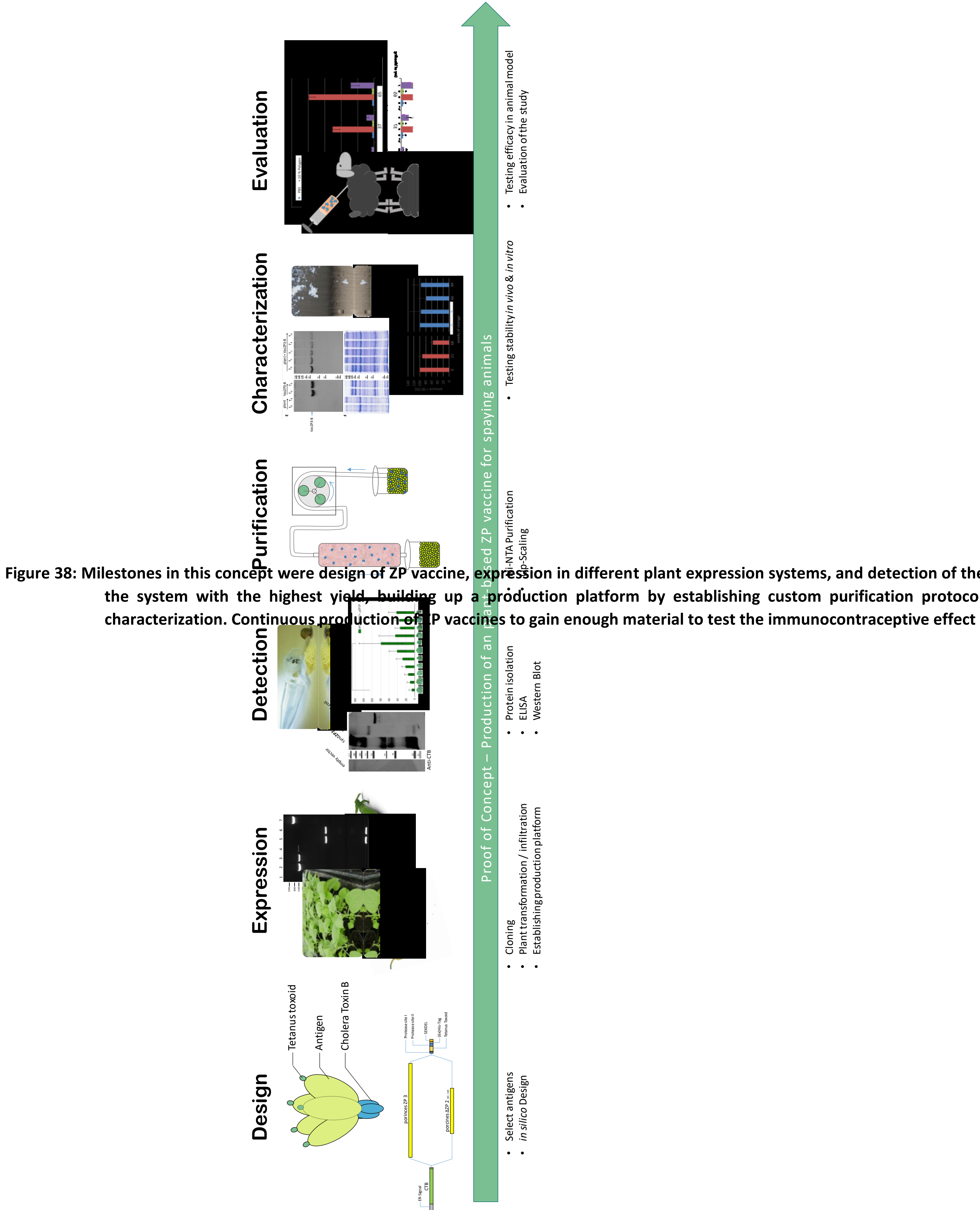


Figure 38: Milestones in this concept were design of ZP vaccine, expression in different plant expression systems, and detection of the the system with the highest yield, building up a production platform by establishing custom purification protoco characterization. Continuous production of ZP vaccines to gain enough material to test the immunocontraceptive effect

6. Summary

The propagation of selected populations of elephants, white-tailed deer and feral horses is regulated by vaccination with porcine oocyte surface proteins. An extension of this method to other wild mammals is limited because of high costs, composed of the production from pig oocytes and the mandatory parenteral injection. Recombinant expressions of Zona Pellucide (ZP) vaccines open the possibility for cheap upscaling. The main aim of the present study was to evaluate the possibility of plant-based production of a ZP vaccine. The methodological approach included different plants gene expression systems to pick the optimal production platform.

In this work the *N*-terminal part of porcine ZP2 and the full-length porcine ZP3 was used as antigens. Both play a crucial role in fertilization and blocking, which further leads to a contraceptive effect in various mammalian species. The antigens are N- and C-terminally fused to the bacterial adjuvants cholera toxin subunit b (CTB) and tetanus toxoid$_{830\text{-}844}$, which could make an additional adjuvant obsolete. The vaccine concentrations in the plant cells were measured by a CTB ELISA.

The expression of VacZP2 and VacZP3-P was evaluated both in constitutive and transient expression systems, because these vaccines featured opposite advantages and disadvantages in production cost and duration. Under the control of the constitutive *arcelin5*-I promoter we detected 0.24 and 0.03 μg_{CTB} mg_{DW}^{-1} for VacZP2 and VacZP3-P in tobacco seeds, respectively. Due to the low enrichment (< 0.6 ng_{CTB} mg_{DW}^{-1}) of VacZP3-P in pea seeds, VacZP2 in carrot cells and tobacco leaves via the constitutive CaMV35S promoter, these production platforms were not practical. The highest yield of both ZP vaccines was achieved by the viral MagnICON system in *N. benthamiana*, which was chosen to produce sufficient amounts for animal testing. The CTB concentration was 5- times higher for VacZP2 and 4-folds higher for VacZP3-P than in the tobacco seed-specific expression. Both vaccines were *N*-glycosylated.

Plant-derived VacZP2 and VacZP3-P showed no decrease in concentration in freeze-dried leaves for at least 35 and 21 weeks, respectively. Since both vaccines exhibit protein degradation after isolation at room temperature, purification under cooled or protease-inhibited conditions was necessary. VacZP2 elicited a high antibody titer in FvB mice even without additional adjuvant. Hamsters showed also a negative effect on fertility. Two out of four females had no offspring in contrast to the control group were all animals bear. VacZP3-P stimulates the immune system of BALB/c mice even with small doses. A subsequent mating experiment showed a dose-dependent spaying effect with an offspring reduction of up to 43 % with the highest dosage.

In summary, porcine ZP2 and ZP3 was accumulated in various plant species and organs via different expression systems. The recombinant expression of these proteins enables a cheap production and easy scale-up in comparison to pig ovaries as the common production platform for porcine antigens.

7. Reference

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V. Supplement

Table 5: PCR program 1

Step		
Denaturation	95 °C	30 sec
Primer Annealing	70 °C	30 sec
Elongation	72 °C	90 sec
Cycles	30	

Table 6: PCR program 2 „Dream60“

Step		
Denaturation	95 °C	30 sec
Primer Annealing	60 °C	30 sec
Elongation	72 °C	90 sec
Cycles	25	

Table 7: PCR program 3 „Colony-PCR“

Step		
Denaturation	95 °C	30 sec
Primer Annealing	55 °C	30 sec
Elongation	72 °C	30 - 90 sec
Cycles	26	

Table 8: PCR program 4

Step		
Denaturierung	95 °C	30 sec
Primer Annealing	60 °C	30 sec
Elongation	72 °C	90 sec
Zyklen	26	

Tabelle 5: PCR Programm 5

Step		
Denaturierung	95 °C	30 sec
Primer Annealing	55 °C	30 sec
Elongation	72 °C	120 sec
Zyklen	30	

Table 9: List of used primer

Name	Sequence
Seq_pICH29912_fw	ACAAACCCGAATCAGTACCAG
Seq_pICH29912_rv	GTGGAGAGAAAAACACTATGC
pZP3_fw	CAGCCTGTGTGGCAAGACGA
pZP3_rev	TGACAATGAAGGAGTGCCGC
Seq_DTB_ER_3'_fw	GTCGAGCTATTGACGGAGAT
Seq_CTB_5'_rev	GGCCAGCGACTCAGTATAAC
Act-fw	GCAACTGGGATGATATGGAGAA
Act-rv	GTGCCTTTGCAATCCACATCTG
Seq_Modul SapI_r	TTAAGTTCGCTAAGTTCTGG
DTB_proof_fw	GCACCCAGAACTTAGCGAAC
DTB_proof_rev	TCCAACGTAAACTGGGCTTT
CTB_prove_fw	TGCTTAGTTCCGCCTATGCT
CTB_prove_rev	GGGGTCTTGTTGTTCCACAC
DTB_re-prove_fw	CGGGCCTATCAAAAACAAGA
DTB_re-prove_rev	TCTGGTATCCGAGAACTCCAA
pZP3_Tabak_fw	ATGGTTGAATGCCAAGAAGC
pZP3_Tabak_rev	ACACGATCAGCAGGGGTAAC
Seq_CTB_Tabak_rev	TCCCTCTTGCCAGCTAAAGA
Seq_3'_synZP3_fw	TGTTGCCATAAGGGACAATG
BsaI_repair_fw	CCCGGGGGTCTC**AC**ATGGCTAGTTC
BsaI_repair_rev	GTCGACGGTCTC**AAAGC**TTACAGTTCGTCTTTCTC
pJET1.2_fw	CGACTCACTATAGGGAGAGCGGC
pJET1.2_rev	AAGAACATCGATTTTCCATGGCAG
ÜEx_synZP3_NdeI	CATATGACTCCCCAGAATATCACTG
ÜEx_synZP3_XhoI	CTCGAGTTACAGTTCGTCTTTCTCT
Seq_synpZP3_r	GATAGATAAGTTTCCTGCTGGTCGC
pET28-fw	TCCCGCGAAATTAATACGAC
pET28-rv	CTTTCGGGCTTTGTTAGCAG
TMV-fw	GATCCGGACGTCGAAGGTTTCGAAGG
TMV-rev	CTTGACTCTAGCTAGAGCGGCCGCTGG
daf9	CCGACGCGGC
CTB_new_fw	CACAAATTCACACTCTTAACGACA
CTB_new_rev	TAGCCATACTTATAGCCGCAATG
pZP2_pICH31120f	GGTCTCAAGGTATAGGGGTCA
pZP2_pICH31120r	GGTCTCAAAGCTCACAGTTCGTCTTTCTCTGAGTGGTGGTGGTGGTG
CTB_new_fw	CACAAATTCACACTCTTAACGACA
CTB_new_rev	TAGCCATACTTATAGCCGCAATG
pZP2_TT_fw	ATAGGGGTCAATCAGTTGGT
pZP2_TT_rev	CGCCATTCCAGGAAAGAAGT

Suppl. 1: Alignment of *vacZP2-P* with consensus sequences for plantal transcription stop. For a transcriptional stop consensus sequences must be in following order: UE (-60 bp), PE (-20 to -30 bp), T-rich (directly before CS), CS, T-rich (n…not found within sequence; f…found within sequence)

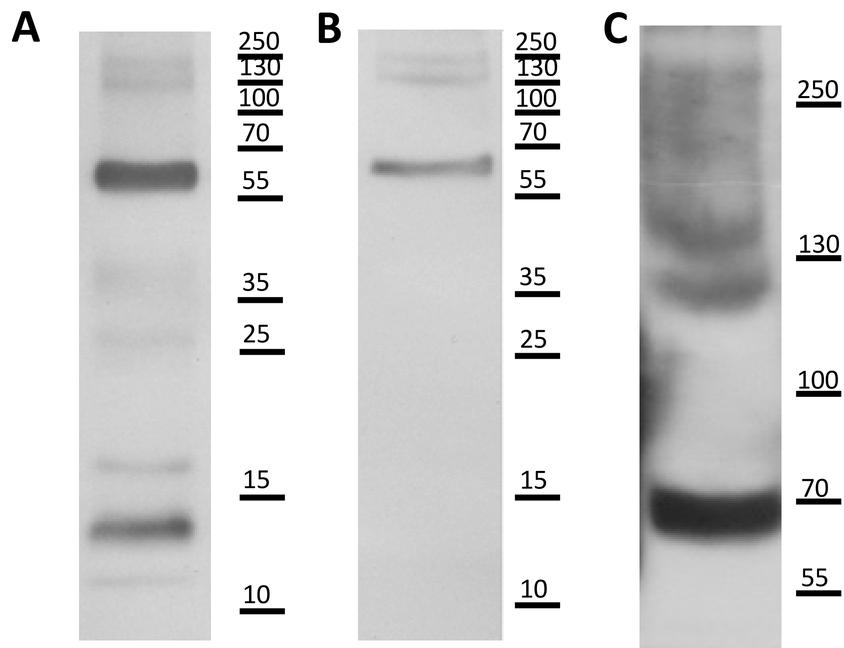
```
                     10         20         30         40         50         60
             ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
VacZP2       ATGGCTAGTT CCATAAAATT GAAATTTGGC GTGTTCTTTA CAGTGCTTTT ATCATCAGCA
ER-Signal    ~~~~~~~~~~ ~~ATAAAATT GAAATTTGGC GTGTTCTTTA CAGTGCTTTT ATCATCAGCA
CTB          ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
GPGP         ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
UE_1_n       TTGTAT---- ---------- ---------- ---------- ---------- ----------
UE_2_n       TTGTAA---- ---------- ---------- ---------- ---------- ----------
UE_3_n       TTTGTA---- ---------- ---------- ---------- ---------- ----------
UE_4_n       TGTTGTG--- ---------- ---------- ---------- ---------- ----------
UE_5_n       TGTGTTTTTT ---------- ---------- ---------- ---------- ----------
PE_1_n       AATAAA---- ---------- ---------- ---------- ---------- ----------
PE_2_f       ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
T-rich_1_f   ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
T-rich_2_n   TTTTTT
CS_1         TA-------- ---------- ---------- ---------- ---------- ----------
CS_2         CA-------- ---------- ---------- ---------- ---------- ----------

                     70         80         90        100        110        120
             ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
VacZP2       TATGCGCATG GTACTCCCCA GAATATCACT GATCTTTGTG CTGAGTATCA TAACACACAA
ER-Signal    TATGCGCATG GT-------- ---------- ---------- ---------- ----------
CTB          ~~~~~~~~~~ ~~ACTCCCCA GAATATCACT GATCTTTGTG CTGAGTATCA TAACACACAA
GPGP         ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
UE_1_n       ---------- ---------- ---------- ---------- ---------- ----------
UE_2_n       ---------- ---------- ---------- ---------- ---------- ----------
UE_3_n       ---------- ---------- ---------- ---------- ---------- ----------
UE_4_n       ---------- ---------- ---------- ---------- ---------- ----------
UE_5_n       ---------- ---------- ---------- ---------- ---------- ----------
PE_1_n       ---------- ---------- ---------- ---------- ---------- ----------
PE_2_f       ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
T-rich_1_f   ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
T-rich_2_n
CS_1         ---------- ---------- ---------- ---------- ---------- ----------
CS_2         ---------- ---------- ---------- ---------- ---------- ----------

                    130        140        150        160        170        180
             ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
VacZP2       ATTCACACTC TTAACGACAA AATCTTCAGC TATACCGAAT CTTTAGCTGG CAAGAGGGAG
ER-Signal    ---------- ---------- ---------- ---------- ---------- ----------
CTB          ATTCACACTC TTAACGACAA AATCTTCAGC TATACCGAAT CTTTAGCTGG CAAGAGGGAG
GPGP         ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
UE_1_n       ---------- ---------- ---------- ---------- ---------- ----------
UE_2_n       ---------- ---------- ---------- ---------- ---------- ----------
UE_3_n       ---------- ---------- ---------- ---------- ---------- ----------
UE_4_n       ---------- ---------- ---------- ---------- ---------- ----------
UE_5_n       ---------- ---------- ---------- ---------- ---------- ----------
PE_1_n       ---------- ---------- ---------- ---------- ---------- ----------
PE_2_f       ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
T-rich_1_f   ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
T-rich_2_n
CS_1         ---------- ---------- ---------- ---------- ---------- ----------
CS_2         ---------- ---------- ---------- ---------- ---------- ----------
```

```
                   190        200        210        220        230        240
               ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
VacZP2         ATGGCAATTA TAACATTCAA GAATGGAGCA ACTTTTCAGG TTGAAGTTCC AGGATCTCAA
ER-Signal      ---------- ---------- ---------- ---------- ---------- ----------
CTB            ATGGCAATTA TAACATTCAA GAATGGAGCA ACTTTTCAGG TTGAAGTTCC AGGATCTCAA
GPGP           ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
UE_1_n         ---------- ---------- ---------- ---------- ---------- ----------
UE_2_n         ---------- ---------- ---------- ---------- ---------- ----------
UE_3_n         ---------- ---------- ---------- ---------- ---------- ----------
UE_4_n         ---------- ---------- ---------- ---------- ---------- ----------
UE_5_n         ---------- ---------- ---------- ---------- ---------- ----------
PE_1_n         ---------- ---------- ---------- ---------- ---------- ----------
PE_2_f         ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
T-rich_1_f     ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
T-rich_2_n
CS_1           ---------- ---------- ---------- ---------- ---------- ----------
CS_2           ---------- ---------- ---------- ---------- ---------- ----------

                   250        260        270        280        290        300
               ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
VacZP2         CACATCGATT CCCAGAAGAA GGCAATTGAG AGAATGAAGG ATACATTGAG GATTGCATAT
ER-Signal      ---------- ---------- ---------- ---------- ---------- ----------
CTB            CACATCGATT CCCAGAAGAA GGCAATTGAG AGAATGAAGG ATACATTGAG GATTGCATAT
GPGP           ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
UE_1_n         ---------- ---------- ---------- ---------- ---------- ----------
UE_2_n         ---------- ---------- ---------- ---------- ---------- ----------
UE_3_n         ---------- ---------- ---------- ---------- ---------- ----------
UE_4_n         ---------- ---------- ---------- ---------- ---------- ----------
UE_5_n         ---------- ---------- ---------- ---------- ---------- ----------
PE_1_n         ---------- ---------- ---------- ---------- ---------- ----------
PE_2_f         ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~AATGAA-- ---------- ----------
T-rich_1_f     ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
T-rich_2_n
CS_1           ---------- ---------- ---------- ---------- ---------- ----------
CS_2           ---------- ---------- ---------- ---------- ---------- ----------

                   310        320        330        340        350        360
               ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
VacZP2         CTCACTGAGG CAAAAGTGGA GAAACTGTGC GTATGGAATA ACAAAACACC CCATGCCATT
ER-Signal      ---------- ---------- ---------- ---------- ---------- ----------
CTB            CTCACTGAGG CAAAAGTGGA GAAACTGTGC GTATGGAATA ACAAAACACC CCATGCCATT
GPGP           ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
UE_1_n         ---------- ---------- ---------- ---------- ---------- ----------
UE_2_n         ---------- ---------- ---------- ---------- ---------- ----------
UE_3_n         ---------- ---------- ---------- ---------- ---------- ----------
UE_4_n         ---------- ---------- ---------- ---------- ---------- ----------
UE_5_n         ---------- ---------- ---------- ---------- ---------- ----------
PE_1_n         ---------- ---------- ---------- ---------- ---------- ----------
PE_2_f         ---------- ---------- ---------- ---------- ---------- ----------
T-rich_1_f     ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
T-rich_2_n
CS_1           ---------- ---------- ---------- ---------- ---------- ----------
CS_2           ---------- ---------- ---------- ---------- ---------- ----------
```

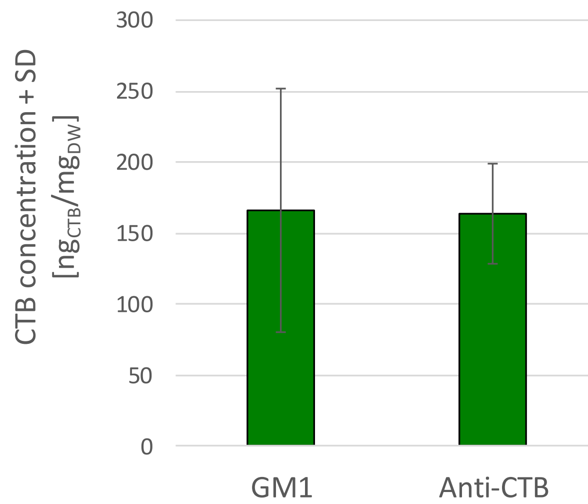
```
                    370        380        390        400        410        420
            ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
VacZP2      GCGGCTATAA GTATGGCTAA TGGTCCAGGA CCACTTAAGA TAGGGGTCAA TCAGTTGGTT
ER-Signal   ---------- ---------- ---------- ---------- ---------- ----------
CTB         GCGGCTATAA GTATGGCTAA T--------- ---------- ---------- ----------
GPGP        ~~~~~~~~~~ ~~~~~~~~~~ ~GGTCCAGGA CCA------- ---------- ----------
UE_1_n      ---------- ---------- ---------- ---------- ---------- ----------
UE_2_n      ---------- ---------- ---------- ---------- ---------- ----------
UE_3_n      ---------- ---------- ---------- ---------- ---------- ----------
UE_4_n      ---------- ---------- ---------- ---------- ---------- ----------
UE_5_n      ---------- ---------- ---------- ---------- ---------- ----------
PE_1_n      ---------- ---------- ---------- ---------- ---------- ----------
PE_2_f      ---------- ---------- ---------- ---------- ---------- ----------
T-rich_1_f  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
T-rich_2_n
CS_1        ---------- ---------- ---------- ---------- ---------- ----------
CS_2        ---------- ---------- ---------- ---------- ---------- ----------

                    430        440        450        460        470        480
            ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
VacZP2      AACACAGCAT TTCCTGGCAT TGTGACTTGT CATGAGAATC GTATGGTTGT GGAGTTTCCG
ER-Signal   ---------- ---------- ---------- ---------- ---------- ----------
CTB         ---------- ---------- ---------- ---------- ---------- ----------
GPGP        ---------- ---------- ---------- ---------- ---------- ----------
UE_1_n      ---------- ---------- ---------- ---------- ---------- ----------
UE_2_n      ---------- ---------- ---------- ---------- ---------- ----------
UE_3_n      ---------- ---------- ---------- ---------- ---------- ----------
UE_4_n      ---------- ---------- ---------- ---------- ---------- ----------
UE_5_n      ---------- ---------- ---------- ---------- ---------- ----------
PE_1_n      ---------- ---------- ---------- ---------- ---------- ----------
PE_2_f      ---------- ---------- ---------- ---------- ---------- ----------
T-rich_1_f  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
T-rich_2_n
CS_1        ---------- ---------- ---------- ---------- ---------- ----------
CS_2        ---------- ---------- ---------- ---------- ---------- ----------

                    490        500        510        520        530        540
            ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
VacZP2      AGAATTCTTG GCACTAAGAT CCAGTATACA TCTGTTGTAG ATCCTTTAGG TTTGGAGATG
ER-Signal   ---------- ---------- ---------- ---------- ---------- ----------
CTB         ---------- ---------- ---------- ---------- ---------- ----------
GPGP        ---------- ---------- ---------- ---------- ---------- ----------
UE_1_n      ---------- ---------- ---------- ---------- ---------- ----------
UE_2_n      ---------- ---------- ---------- ---------- ---------- ----------
UE_3_n      ---------- ---------- ---------- ---------- ---------- ----------
UE_4_n      ---------- ---------- ---------- ---------- ---------- ----------
UE_5_n      ---------- ---------- ---------- ---------- ---------- ----------
PE_1_n      ---------- ---------- ---------- ---------- ---------- ----------
PE_2_f      ---------- ---------- ---------- ---------- ---------- ----------
T-rich_1_f  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
T-rich_2_n
CS_1        ---------- ---------- ---------- ---------- ---------- ----------
CS_2        ---------- ---------- ---------- ---------- ---------- ----------
```

```
                    550       560       570       580       590       600
            ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
VacZP2      ATGAACTGTA CTTACGTCCT TGATCCCGAA AATCTGACTC TCAAAGCTCC ATATGAAGCA
ER-Signal   ---------- ---------- ---------- ---------- ---------- ----------
CTB         ---------- ---------- ---------- ---------- ---------- ----------
GPGP        ---------- ---------- ---------- ---------- ---------- ----------
UE_1_n      ---------- ---------- ---------- ---------- ---------- ----------
UE_2_n      ---------- ---------- ---------- ---------- ---------- ----------
UE_3_n      ---------- ---------- ---------- ---------- ---------- ----------
UE_4_n      ---------- ---------- ---------- ---------- ---------- ----------
UE_5_n      ---------- ---------- ---------- ---------- ---------- ----------
PE_1_n      ---------- ---------- ---------- ---------- ---------- ----------
PE_2_f      ---------- ---------- ---------- ---------- ---------- ----------
T-rich_1_f  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
T-rich_2_n
CS_1        ---------- ---------- ---------- ---------- ---------- ----------
CS_2        ---------- ---------- ---------- ---------- ---------- ----------

                    610       620       630       640       650       660
            ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
VacZP2      TGCACAAAGA GAGTTAGAGG ACATCACCAA ATGACGATTA GGCTAATCGA CGATAATGCA
ER-Signal   ---------- ---------- ---------- ---------- ---------- ----------
CTB         ---------- ---------- ---------- ---------- ---------- ----------
GPGP        ---------- ---------- ---------- ---------- ---------- ----------
UE_1_n      ---------- ---------- ---------- ---------- ---------- ----------
UE_2_n      ---------- ---------- ---------- ---------- ---------- ----------
UE_3_n      ---------- ---------- ---------- ---------- ---------- ----------
UE_4_n      ---------- ---------- ---------- ---------- ---------- ----------
UE_5_n      ---------- ---------- ---------- ---------- ---------- ----------
PE_1_n      ---------- ---------- ---------- ---------- ---------- ----------
PE_2_f      ---------- ---------- ---------- ---------- ---------- ----------
T-rich_1_f  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
T-rich_2_n
CS_1        ---------- ---------- ---------- ---------- ---------- ----------
CS_2        ---------- ---------- ---------- ---------- ---------- ----------

                    670       680       690       700       710       720
            ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
VacZP2      GCTTTACGAC AAGAAGCCCT TATGTACCAC ATAAGTTGTC CAGTAATGGG TGCTGAAGGT
ER-Signal   ---------- ---------- ---------- ---------- ---------- ----------
CTB         ---------- ---------- ---------- ---------- ---------- ----------
GPGP        ---------- ---------- ---------- ---------- ---------- ----------
UE_1_n      ---------- ---------- ---------- ---------- ---------- ----------
UE_2_n      ---------- ---------- ---------- ---------- ---------- ----------
UE_3_n      ---------- ---------- ---------- ---------- ---------- ----------
UE_4_n      ---------- ---------- ---------- ---------- ---------- ----------
UE_5_n      ---------- ---------- ---------- ---------- ---------- ----------
PE_1_n      ---------- ---------- ---------- ---------- ---------- ----------
PE_2_f      ---------- ---------- ---------- ---------- ---------- ----------
T-rich_1_f  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
T-rich_2_n
CS_1        ---------- ---------- ---------- ---------- ---------- ----------
CS_2        ---------- ---------- ---------- ---------- ---------- ----------
```

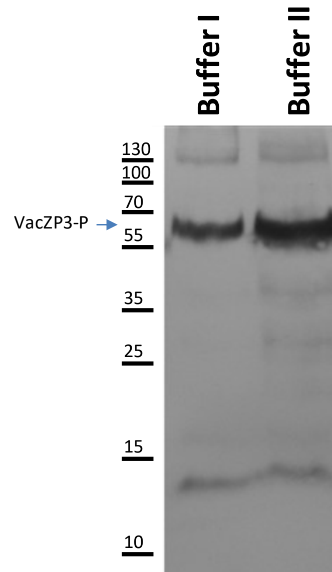
```
                    730        740        750        760        770        780
            ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
VacZP2      CCAGATCAAC ATTCAGGATC TACCATTTGC ATGAAAGACT TCATGTCCTT TACCTTCAAC
ER-Signal   ---------- ---------- ---------- ---------- ---------- ----------
CTB         ---------- ---------- ---------- ---------- ---------- ----------
GPGP        ---------- ---------- ---------- ---------- ---------- ----------
UE_1_n      ---------- ---------- ---------- ---------- ---------- ----------
UE_2_n      -
UE_3_n      ---------- ---------- ---------- ---------- ---------- ----------
UE_4_n      ---------- ---------- ---------- ---------- ---------- ----------
UE_5_n      ---------- ---------- ---------- ---------- ---------- ----------
PE_1_n      ---------- ---------- ---------- ---------- ---------- ----------
PE_2_f      ---------- ---------- ---------- ---------- ---------- ----------
T-rich_1_f  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
T-rich_2_n
CS_1        ---------- ---------- ---------- ---------- ---------- ----------
CS_2        ---------- ---------- ---------- ---------- ---------- ----------

                    790        800        810        820        830        840
            ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
VacZP2      TTCTTTCCTG GAATGGCGTG TACAATTGAA GGAAGAAAGA AGCAGTACAT TAAAGCCAAT
ER-Signal   ---------- ---------- ---------- ---------- ---------- ----------
CTB         ---------- ---------- ---------- ---------- ---------- ----------
GPGP        ---------- ---------- ---------- ---------- ---------- ----------
UE_1_n      ---------- ---------- ---------- ---------- ---------- ----------
UE_2_n
UE_3_n      ---------- ---------- ---------- ---------- ---------- ----------
UE_4_n      ---------- ---------- ---------- ---------- ---------- ----------
UE_5_n      ---------- ---------- ---------- ---------- ---------- ----------
PE_1_n      ---------- ---------- ---------- ---------- ---------- ----------
PE_2_f      ---------- ---------- ---------- ---------- ---------- ----------
T-rich_1_f  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ TTTTCT---- ----------
T-rich_2_n
CS_1        ---------- ---------- ---------- ---------- ---------- ----------
CS_2        ---------- ---------- ---------- ---------- ---------- ----------

                    850        860        870        880        890        900
            ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
VacZP2      AGCAAGTTTA TAGGGATAAC CGAACTAGAG AACCTGTACT TTCAAGGTGG ATCCCATCAT
ER-Signal   ---------- ---------- ---------- ---------- ---------- ----------
CTB         ---------- ---------- ---------- ---------- ---------- ----------
GPGP        ---------- ---------- ---------- ---------- ---------- ----------
UE_1_n      ---------- ---------- ---------- ---------- ---------- ----------
UE_2_n
UE_3_n      ---------- ---------- ---------- ---------- ---------- ----------
UE_4_n      ---------- ---------- ---------- ---------- ---------- ----------
UE_5_n      ---------- ---------- ---------- ---------- ---------- ----------
PE_1_n      ---------- ---------- ---------- ---------- ---------- ----------
PE_2_f      ---------- ---------- ---------- ---------- ---------- ----------
T-rich_1_f  ---------- ---------- ---------- ---------- ---------- ----------
T-rich_2_n
CS_1        ---------- ---------- ---------- ---------- ---------- ----------
CS_2        ---------- ---------- ---------- ---------- ---------- ----------

                    910        920        930        940        950        960
            ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
VacZP2      CACCATCACC ACTCAGAGAA AGACGAACTG TAA------- ---------- ----------
ER-Signal   ---------- ---------- ---------- ---------- ---------- ----------
CTB         ---------- ---------- ---------- ---------- ---------- ----------
GPGP        ---------- ---------- ---------- ---------- ---------- ----------
UE_1_n      ---------- ---------- ---------- ---------- ---------- ----------
UE_2_n
UE_3_n      ---------- ---------- ---------- ---------- ---------- ----------
UE_4_n      ---------- ---------- ---------- ---------- ---------- ----------
UE_5_n      ---------- ---------- ---------- ---------- ---------- ----------
```



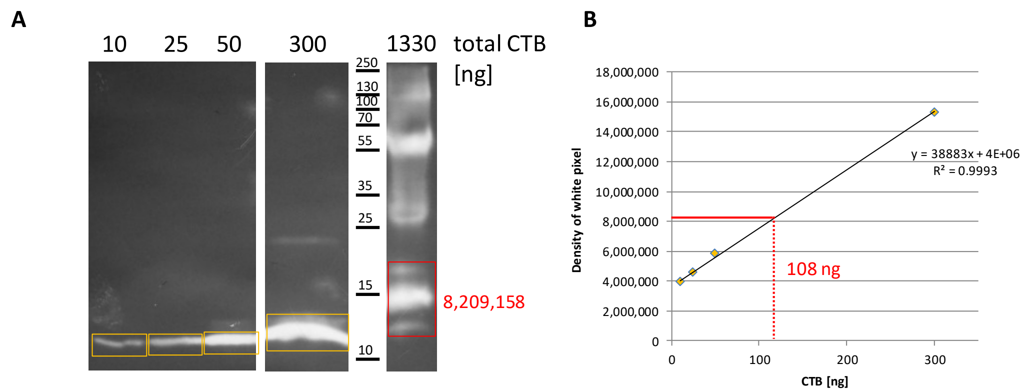
Suppl. 2: Western blot analysis of VacZP3-P. (A) Anti-CTB and (B) anti-histidine Western blot analysis of VacZP3-P in crude *N. benthamiana* protein extract, separated in a 12 % SDS-PAGE. (C) Anti-CTB Western blot analysis of VacZP3-P in crude tobacco protein extract, separated in a 6 % SDS-PAGE.



Suppl. 3: GM1 and anti-CTB binding assay of VacZP3-P (n = 2)



Suppl. 4: ***N. benthamiana*** **protein extract of transiently expressed VacZP3-P detected in anti-CTB Western blot analysis. Buffer I: Protein extraction buffer without protease inhibitor mix (see material & methods); Buffer II: Davoodi-Semiromi et al. 2010**



Suppl. 5: Densitometric analysis of free CTB molecules in purified VacZP3-P (in 1 x PBS pH 7.2). (A) Digitally colour-inverted and desaturated Anti-CTB Western blot analysis. Lane 1...10 ng CTB Standard; 2...25 ng CTB; 3...50 ng CTB; 4...300 ng CTB, 5...1330 ng VacZP3-P (determined in anti-CTB ELISA). (B) Standard curve of white pixel densities from CTB standard (yellow).

Suppl. 6: Alignment of *vacZP3-P* with consensus sequences for plantal transcription stop. For a transcriptional stop consensus sequences must be in following order: UE (-60 bp), PE (-20 to -30 bp), T-rich (directly before CS), CS, T-rich (n…not found within sequence; f…found within sequence)

```
                       10         20         30         40         50         60
               ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
VacZP3         ATGGCTAGTT CCATAAAATT GAAATTTGGC GTGTTCTTTA CAGTGCTTTT ATCATCAGCA
ER-Signal      ~~~~~~~~~~ ~~ATAAAATT GAAATTTGGC GTGTTCTTTA CAGTGCTTTT ATCATCAGCA
CTB            ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
GPGP           ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
UE_1_n         TTGTAT
UE_2_f         ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
UE_3_n         TTTGTA
UE_4_n         TGTTGTG
UE_5_n         TGTGTTTTTT
PE_1_n         AATAAA
PE_2_f         ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
T-rich_1_f     ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
T-rich_2_n     TTTTTT
CS_1           TA
CS_2           CA

                       70         80         90        100        110        120
               ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
VacZP3         TATGCGCATG GTACTCCCCA GAATATCACT GATCTTTGTG CTGAGTATCA TAACACACAA
ER-Signal      TATGCGCATG GT
CTB            ~~~~~~~~~~ ~~ACTCCCCA GAATATCACT GATCTTTGTG CTGAGTATCA TAACACACAA
GPGP           ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
UE_1_n
UE_2_f         ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
UE_3_n
UE_4_n
UE_5_n
PE_1_n
PE_2_f         ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
T-rich_1_f     ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
T-rich_2_n
CS_1
CS_2

                      130        140        150        160        170        180
               ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
VacZP3         ATTCACACTC TTAACGACAA AATCTTCAGC TATACCGAAT CTTTAGCTGG CAAGAGGGAG
ER-Signal
CTB            ATTCACACTC TTAACGACAA AATCTTCAGC TATACCGAAT CTTTAGCTGG CAAGAGGGAG
GPGP           ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
UE_1_n
UE_2_f         ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
UE_3_n
UE_4_n
UE_5_n
PE_1_n
PE_2_f         ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
T-rich_1_f     ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
T-rich_2_n
CS_1
CS_2
```

```
                    190        200        210        220        230        240
             ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
VacZP3       ATGGCAATTA TAACATTCAA GAATGGAGCA ACTTTTCAGG TTGAAGTTCC AGGATCTCAA
ER-Signal
CTB          ATGGCAATTA TAACATTCAA GAATGGAGCA ACTTTTCAGG TTGAAGTTCC AGGATCTCAA
GPGP         ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
UE_1_n
UE_2_f       ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
UE_3_n
UE_4_n
UE_5_n
PE_1_n
PE_2_f       ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
T-rich_1_f   ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
T-rich_2_n
CS_1
CS_2

                    250        260        270        280        290        300
             ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
VacZP3       CACATCGATT CCCAGAAGAA GGCAATTGAG AGAATGAAGG ATACATTGAG GATTGCATAT
ER-Signal
CTB          CACATCGATT CCCAGAAGAA GGCAATTGAG AGAATGAAGG ATACATTGAG GATTGCATAT
GPGP         ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
UE_1_n
UE_2_f       ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
UE_3_n
UE_4_n
UE_5_n
PE_1_n
PE_2_f       ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~AATGAA
T-rich_1_f   ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
T-rich_2_n
CS_1
CS_2

                    310        320        330        340        350        360
             ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
VacZP3       CTCACTGAGG CAAAAGTGGA GAAACTGTGC GTATGGAATA ACAAAACACC CCATGCCATT
ER-Signal
CTB          CTCACTGAGG CAAAAGTGGA GAAACTGTGC GTATGGAATA ACAAAACACC CCATGCCATT
GPGP         ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
UE_1_n
UE_2_f       ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
UE_3_n
UE_4_n
UE_5_n
PE_1_n
PE_2_f
T-rich_1_f   ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
T-rich_2_n
CS_1
CS_2
```

```
                      370        380        390        400        410        420
              ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
VacZP3        GCGGCTATAA GTATGGCTAA TGGTCCAGGA CCACTTAAGC AGCCTGTCTG GCAAGACGAA
ER-Signal
CTB           GCGGCTATAA GTATGGCTAA T
GPGP          ~~~~~~~~~~ ~~~~~~~~~~ ~GGTCCAGGA CCA
UE_1_n
UE_2_f        ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
UE_3_n
UE_4_n
UE_5_n
PE_1_n
PE_2_f
T-rich_1_f    ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
T-rich_2_n
CS_1
CS_2

                      430        440        450        460        470        480
              ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
VacZP3        GGTCAGAGAT TACGACCTAG TAAGCCTCCG ACAGTTATGG TTGAATGCCA AGAAGCCCAA
ER-Signal
CTB
GPGP
UE_1_n
UE_2_f        ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
UE_3_n
UE_4_n
UE_5_n
PE_1_n
PE_2_f
T-rich_1_f    ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
T-rich_2_n
CS_1
CS_2

                      490        500        510        520        530        540
              ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
VacZP3        TTGGTCGTGA TTGTCTCTAA GGACTTATTT GGCACTGGTA AACTGATTCG TCCAGCAGAT
ER-Signal
CTB
GPGP
UE_1_n
UE_2_f        ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
UE_3_n
UE_4_n
UE_5_n
PE_1_n
PE_2_f
T-rich_1_f    ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
T-rich_2_n
CS_1
CS_2
```

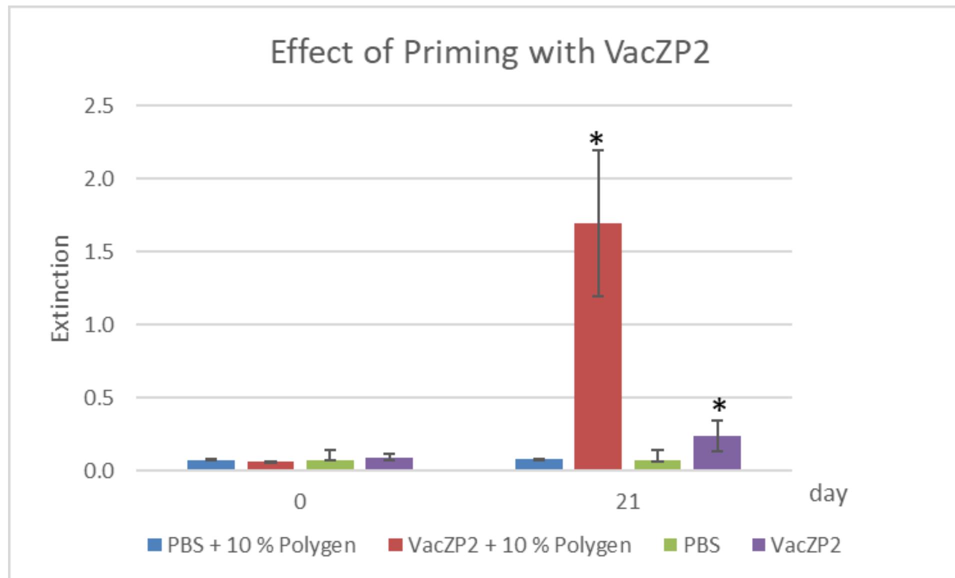
```
                      550        560        570        580        590        600
              ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
VacZP3        TTGAGCCTTG GACCAGCCAA ATGTGAACCC CTAGTCTCAC AAGATACCGA TGCAGTGGTT
ER-Signal
CTB
GPGP
UE_1_n
UE_2_f        ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
UE_3_n
UE_4_n
UE_5_n
PE_1_n
PE_2_f
T-rich_1_f    ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
T-rich_2_n
CS_1
CS_2

                      610        620        630        640        650        660
              ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
VacZP3        AGGTTCGAAG TAGGGCTTCA TGAATGTGGT TCTTCATTGC AAGTTACGGA TGATGCCCTT
ER-Signal
CTB
GPGP
UE_1_n
UE_2_f        ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
UE_3_n
UE_4_n
UE_5_n
PE_1_n
PE_2_f
T-rich_1_f    ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
T-rich_2_n
CS_1
CS_2

                      670        680        690        700        710        720
              ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
VacZP3        GTTTACTCTA CCTTCTTGAG ACACGATCCG CGACCAGCAG GAAACTTATC TATCTTGAGG
ER-Signal
CTB
GPGP
UE_1_n
UE_2_f        ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
UE_3_n
UE_4_n
UE_5_n
PE_1_n
PE_2_f
T-rich_1_f    ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
T-rich_2_n
CS_1
CS_2
```

```
                       730        740        750        760        770        780
               ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
VacZP3         ACAAATAGGG CAGAAGTGCC TATCGAGTGC CATTATCCTA GACAAGGCAA TGTTAGTAGT
ER-Signal
CTB
GPGP
UE_1_n
UE_2_f         ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
UE_3_n
UE_4_n
UE_5_n
PE_1_n
PE_2_f
T-rich_1_f     ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
T-rich_2_n
CS_1
CS_2

                       790        800        810        820        830        840
               ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
VacZP3         TGGGCCATTC TGCCAACATG GGTACCGTTT CGTACGACCG TTTTCTCTGA GGAGAAACTC
ER-Signal
CTB
GPGP
UE_1_n
UE_2_f         ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
UE_3_n
UE_4_n
UE_5_n
PE_1_n
PE_2_f
T-rich_1_f     ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ TTTTCT
T-rich_2_n
CS_1
CS_2

                       850        860        870        880        890        900
               ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
VacZP3         GTCTTTTCAT TGAGATTGAT GGAGGAAAAT TGGTCAGCTG AGAAGATGAC TCCAACATTT
ER-Signal
CTB
GPGP
UE_1_n
UE_2_f         ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
UE_3_n
UE_4_n
UE_5_n
PE_1_n
PE_2_f
T-rich_1_f
T-rich_2_n
CS_1
CS_2
```

```
                      910        920        930        940        950        960
              ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
VacZP3        CAGCTTGGGG ACAGAGCTCA TCTTCAGGCT CAAGTCCATA CTGGTTCACA CGTTCCTCTA
ER-Signal
CTB
GPGP
UE_1_n
UE_2_f        ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
UE_3_n
UE_4_n
UE_5_n
PE_1_n
PE_2_f
T-rich_1_f
T-rich_2_n
CS_1
CS_2

                      970        980        990       1000       1010       1020
              ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
VacZP3        AGACTCTTTG TTGACCATTG CGTTGCTACA CTCACTCCAG ATTGGAACAC TAGTCCTTCT
ER-Signal
CTB
GPGP
UE_1_n
UE_2_f        ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
UE_3_n
UE_4_n
UE_5_n
PE_1_n
PE_2_f
T-rich_1_f
T-rich_2_n
CS_1
CS_2

                     1030       1040       1050       1060       1070       1080
              ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
VacZP3        CATACGATCG TAGACTTTCA CGGGTGTTTA GTAGATGGCC TAACTGAAGC TTCTTCTGCA
ER-Signal
CTB
GPGP
UE_1_n
UE_2_f        ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
UE_3_n
UE_4_n
UE_5_n
PE_1_n
PE_2_f
T-rich_1_f
T-rich_2_n
CS_1
CS_2
```

```
                   1090       1100       1110       1120       1130       1140
            ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
VacZP3      TTCAAAGCGC CTAGACCTGG TCCAGAGACT CTCCAATTCA CTGTGGATGT GTTCCACTTT
ER-Signal
CTB
GPGP
UE_1_n
UE_2_f      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
UE_3_n
UE_4_n
UE_5_n
PE_1_n
PE_2_f
T-rich_1_f
T-rich_2_n
CS_1
CS_2

                   1150       1160       1170       1180       1190       1200
            ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
VacZP3      GCTAATGATT CTAGAAACAC AATATACATT ACCTGTCATC TTAAAGTTAC CCCTGCTGAT
ER-Signal
CTB
GPGP
UE_1_n
UE_2_f      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
UE_3_n
UE_4_n
UE_5_n
PE_1_n
PE_2_f
T-rich_1_f
T-rich_2_n
CS_1
CS_2

                   1210       1220       1230       1240       1250       1260
            ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
VacZP3      CGTGTTCCCG ATCAGCTTAA TAAGGCTTGT TCTTTTAGCA AATCCAGCAA TAGGTGGAGT
ER-Signal
CTB
GPGP
UE_1_n
UE_2_f      ~~~~~~~~~~ ~TTGTAA
UE_3_n
UE_4_n
UE_5_n
PE_1_n
PE_2_f
T-rich_1_f
T-rich_2_n
CS_1
CS_2
```

```
                    1270       1280       1290       1300       1310       1320
            ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
VacZP3      CCAGTAGAAG GTCCAGCTGT GATTTGCAGA TGTTGCCATA AGGGACAATG TGGAACTCCT
ER-Signal
CTB
GPGP
UE_1_n
UE_2_f
UE_3_n
UE_4_n
UE_5_n
PE_1_n
PE_2_f
T-rich_1_f
T-rich_2_n
CS_1
CS_2

                    1330       1340       1350       1360       1370       1380
            ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
VacZP3      TCCTTGTCAT GTACAATTGA AGGAAGAAAG AAGCAGTACA TTAAAGCCAA TAGCAAGTTT
ER-Signal
CTB
GPGP
UE_1_n
UE_2_f
UE_3_n
UE_4_n
UE_5_n
PE_1_n
PE_2_f
T-rich_1_f
T-rich_2_n
CS_1
CS_2

                    1390       1400       1410       1420       1430       1440
            ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
VacZP3      ATAGGGATAA CCGAACTAGA GAACCTGTAC TTTCAAGGTG GATCCCATCA TCACCATCAC
ER-Signal
CTB
GPGP
UE_1_n
UE_2_f
UE_3_n
UE_4_n
UE_5_n
PE_1_n
PE_2_f
T-rich_1_f
T-rich_2_n
CS_1
CS_2

                    1450       1460
            ....|....| ....|....| ....
VacZP3      CACTCAGAGA AAGACGAACT GTAA
ER-Signal
CTB
GPGP
UE_1_n
UE_2_f
UE_3_n
UE_4_n
UE_5_n
PE_1_n
PE_2_f
T-rich_1_f
T-rich_2_n
CS_1
CS_2
```



Suppl. 7: Antibody titer of mice immunized with VacZP2 after 0 and 21 days. Primary vaccination was done on day 2. Sera were diluted 1:100. Error bars show standard deviation. Asterisk show significant difference ($p < 0.05$, Tukey-HSD)

VI. Authors contribution

Parts of this thesis was conducted not by myself. Cloning, transformation (stable and transient) and measurements of accumulation level of VacZP2 was conducted by Katharina Unkel, a master student under my supervision. Exception is the transformation and VacZP2 expression in carrot cells, which was done by Robert Krenzlin under the supervision of Dr. Jana Huckauf. Animal trials were done by BioServ`s team around Dr. Pritzsch, especially Juliane Klüß. They supported me by extracting ZP vaccine from plant material. They conducted the immunization, measurements of antibody titer and histological analysis of the ovar. Dr. Jennifer Schön from FBN Dummerstorf also examined the ovarian tissue and gave her assessment about the vaccines' influence on the ovar.

VII. Declaration of independence

I herewith declare that I wrote and composed the presented PhD thesis independently. I did not use any other sources, figures or resources than the ones stated in the references.

Hiermit erkläre ich, dass ich die vorliegende Arbeit selbstständig und ohne, außer die von mir angegebenen, Hilfsmittel angefertigt habe. Alle genutzten Quellen und Zitate sind als solche gekennzeichnet.

Axel Masloboy

Rostock, 19.03.2019

VIII. Publikationen und Tagungsbeiträge

Publikationen

Hasse, Dirk; Andersson, Evalena; Carlsson, Gunilla; Masloboy, Axel; Hagemann, Martin; Bauwe, Hermann; Andersson, Inger (2013): Structure of the homodimeric glycine decarboxylase P-protein from Synechocystis sp. PCC 6803 suggests a mechanism for redox regulation. In: *Journal of Biological Chemistry* 288 (49), S. 35333–35345.

Tagungsbeiträge

- Konferenz der Gesselltschaft für Molecular Farming in Berlin (2014) Poster: Masloboy A, Broer I und Huckauf J, "Plant-made Spaying Vaccine"
- Agrosnet-Doktorandentag in Berlin (2015) Poster: Masloboy A, Broer I und Huckauf J, „Spaying with the Aid of Plant-made Vaccines"
- 3. Konferenz der Gesselltschaft für Molecular Farming in Gent (2016) Short-Talk: Masloboy A, Broer I und Huckauf J, "Spaying with the Aid of Plant-made Vaccines"

IX. Curriculum vitae

Axel Masloboy, Dipl.-Biol.

Personal information

Adress:	Peterskampweg 61 22089 Hamburg Germany
Email:	axel.masloboy@posteo.de
Date of birth:	10/17/1985 in Stralsund/Germany

Professional experience

2013 - 2016 Research associate at the professorship for Agrobiotechnology, agricultural- and environmental faculty, University of Rostock/Germany

- Project management and coordination of tasks with project partners
- Design, conduction, evaluation and publication of experiments
- Transient and stable expression of recombinant proteins in plants and *E. coli*
- Supervision of a master student

2011 - 2013 Research associate at the professorship for Plantphysiology, faculty for mathematics and natural science, University of Rostock/Germany

- Site-directed mutagenesis of the p-protein
- cloning, expression, purification from *E. coli*
- measurements of the catalytic activity of enzyme with radiolabeled substrate

2005 - 2006 Military service as navigator at the speed boat „Ozelot“ in Rostock

Educational profile

2013 - Today	PhD studies at the professorship Agrobiotechnology, Agricultural- and Environmental Faculty University of Rostock/Germany with the topic: „Two Zona Pellucida Vaccines Expressed in Different Plant Expression Systems for Spaying Mammals“ (expected graduation Q3/2018)
2006 – 2011	Diploma studies in biology, University of Rostock/Germany

- Diploma examinations [with grade]: plant physiology [1.3], biochemistry [1.0], ecology [1.7], bioorganic chemistry [1.3]
- Diploma thesis: Genetische und physiologische Charakterisierung von *Arabidopsis*-Linien mit cyanobakteriellen Genen für alternativen Glykolatabbau, Supervisor: Prof. Martin Hagemann, grade: 1.3

1997 - 2005	Qualification for the university entrance, „Abitur“ at the Hansa Gymnasium Stralsund/Germany

Methods

- Cloning, modification and analysis of DNA
- Transformation of bacteria
- Transient and stable transformation of plants (tobacco and pea)
- In soil and in vitro cultivation of plants and selection of transformants
- Analysis of transgenic plants (DNA, RNA, Proteom)
- Expression, isolation and analysis of recombinant proteins in plants and bacteria
- Design, implementation, evaluation and publication of plant related research

Advanced education

01/25/2018	Project management, 2-month seminar
11/21/2017	DEKRA certified quality management representative according to DIN EN ISO 9001:2015, 1-month seminar

Languages

German	First language
English	Fluent
Latin	basics (high school class 7-12)