FINAL REPORT

(07/2011-12/2012)

Project title: Expressing multiple T-cell epitopes in planta for specific immunotherapy

By: **Dr. Lien Quynh Le**
Plant Cell Department
Institute of Biotechnology, Hanoi, Vietnam
Supervisor: **Prof. G.Angenon**
Laboratory of Plant Genetics
Institute for Molecular Biology and Biotechnology
Vrije Universiteit Brussel (VUB)

1. Introduction

Plant molecular farming refers to the production of recombinant proteins including pharmaceutical, industrial proteins or other secondary metabolites in plants (Ahmad et al., 2012). Since the first plant-derived pharmaceutical proteins produced in transgenic tobacco plants in 1986, thirty years later transgenic plants are gaining more and more attention as the new generation of bio-reactors which active research and development activities in molecular farming identified in over one hundred companies, universities and research institutes around the world (Basaran and Rodríguez-Cerezo, 2008). Compared to other expression systems, plant produced recombinant proteins are particularly attractive since they are economical to produce, easily adaptable to large-scale production, present a minimal risk of contamination with animal or human pathogens, and are stable at room temperature for a long time allowing them to be used in remote areas (Walmsley and Arntzen, 2003). Among the pharmaceutical products, the vaccines that one can eat or edible vaccines which is an alternative strategy for vaccine administration to trigger both mucosal and humoral immune responses is now being emerged and developed (Mishra et al., 2008). Transgenic plants are now recognized as legitimate sources for production of biopharmaceuticals, and substantial R&D efforts are devoted to it (Ma et al., 2005), especially oral vaccines because their products have proved to elicit immune responses when fed to animals or humans (Lamphear et al., 2002; Fischer et al. 2004). More than 30 different vaccines produced in more than 10 plant species have been reported (Streatfield and Howard, 2003) and several of these are in clinical trials (Ma et al., 2005).

Allergy to birch (*Betula pendula, syn. B. verrucosa*) is one of the most common pollinosis in Europe and prevalence of birch pollen sensitization has continuously increased year by year. Comprehensive studies on birch pollen allergy identified two birch pollen allergens, Bet v 1 and Bet v 2 (www.allergen.org). Bet v 1 is responsible for IgE binding of more than 95% of birch allergic patients; therefore Bet v 1 is considered as the major allergen of birch pollen allergy (Jarolim et al, 1989). Bet v 1 is encoded by a multigene family containing approximately 30 isoforms in which the similarities are rather high. Especially, the Bet v 1 family shows high identities to PR-10 protein, which can be found in many plant-derived foods such as apples, carrots, tomatoes,… (Rashid et al., , 2011).

Therefore, Bet v 1 is identified as one of the most important elicitors of pollen-related food allergies. Due to cross reaction, pollen allergic patients could develop allergic reactions to many plant-derived foods and vice versa. Bet v 1 is one of the first allergens to be formulated as a recombinant human allergy vaccine. The rBet v 1 produced by Syallergenes SA and used as a biological reference preparation is based on the Bet v 1 1.0201 isoform which is the most abundant one found in birch pollen extracts (Swoboda et al., 1995). Besides, two genetically modified Bet v 1 hypoallergens with a reduced capacity binding to IgE but retained immunogenicity were investigated. These hypoallergenic variants were indicated to reduce the risk of treatment induced immediate allergic reactions. Both recombinant naturally or hypoallergenic derivatives of Bet v 1 have been applied in a number of human allergy vaccine studied and induced strong de novo immunoglobulin (IgG1, IgG2 and IgG4) responses. Furthermore, the induced IgG4 antibodies were shown to prevent Bet v 1-mediated allergic symptoms (Groenlund and Gafvelin, 2010). This phenomenon was explained by the immune deviation of allergen specific T_{H2} cells to a T_{H1} cell-driven response resulting in the inhibition of overproduction of IgE antibodies from B cells (Larché M, 2006; Larché M, 2006). Recently, immunotherapy using T-cell epitopes instead of the whole molecule is being developed. T-cell epitopes are small amino acid fragments which are presented in the context of major histocompatibility class II molecules on the surface of Antigen Presenting Cells and subsequently recognized by T cells. The interaction of these peptides and T cells stimulates naïve T_H cells to become allergen-specific T_{H1} and T_{H2} cells which the balance of these cells finally lead to the overproduction of IgE antibodies from B cells (Roitt I, Brostoff J and Male D, 1993).

Thus, detection of T cell epitopes on allergens could provide us with new aspects for therapy of type I allergic diseases. Additionally, differs from epitopes of other immune cells which are mostly in conformational forms, only linear amino acid sequences are important for T-cell recognition (Meno, 2011). Therefore, immunotherapy using T-cell epitopes could be an easy-established and effective strategy to induce adaptive immune response against allergy.

The aim of this study is to produce the edible T-cell epitopes of the major birch pollen allergen Bet v 1 via transgenic *A.thaliana* plants. In final, we tend to provide a proof of concept for using T-cell epitopes for immunotherapy against the inhaled birch pollen allergy.

2. Materials and methods

2.1. Scanning for Bet v 1 T-cell epitopes

The key word “birch pollen allergy+Bet v 1” was used to search for all isoforms of pollen Bet v 1 in allergen database (www.allergen.org). Homologous of these sequences was performed via MegAlign method (Cluster W, DNAStar). The T-cell epitopes were identified based on literature reviews.

2.2. Vector construction

To express the Bet v 1 T-cell epitopes in *Arabidopsis* transgenic plants, we firstly artificially synthesized epitopes and then and linked to heat-labile enterotoxin B subunit (LTB) from *E. coli* or cholera toxin subunit B (CTB) fragment using the Splicing by Overlap Extension (SOEing) method (Horton et al., 1989 and 1990). The LTB/CTB-epi fragments were then introduced into the pUCN-LTB_PLAN12_3’arc vector using *SalI/XbaI* sites resulting to pUCN-LTB/CTB_epi_3’arc. Beside the

expression of LTB or CTB to increase the mucosal immune responses, these vectors also contain the non-translation region 3'arc to enhance the protein expression level. The LTB/CTB_epi_3'arc fragment was then excised and ligated into pGW2 vector (Department of Plant Genetics, VUB, Belgium) containing the Cre/lox-intron via *ApaI/HpaI* sites to perform the pGW2- epifull vectors. The SDS promoter was then finally introduced into the pGW2- epifull via Gateway cloning strategy (Invitrogen, USA). The resulting vectors were designated as pGW2- epifull/SDS under the control of seed-specific promoter Phasoline promoter. Cloning strategy for these vectors was further described in Fig. 1.

2.3. Transformation of destination vectors into Agrobacterium tumafaciens

Following the cloning strategies, final vectors including:

- pGW2_LTB/2Epi_SDS containing two epitopes Bet v 1 $_{141\text{-}156}$ and Bet v 1 $_{51\text{-}68}$ fused with LTB
- pGW2_LTB/1Epi_SDS containing epitope Bet v 1 $_{141\text{-}156}$ fused with LTB
- pGW2_CTB/2Epi_SDS containing two epitopes Bet v 1 $_{141\text{-}156}$ and Bet v 1 $_{51\text{-}68}$ fused with CTB
- pGW2_CTB/1Epi_SDS containing two epitopes Bet v 1 $_{141\text{-}156}$ fused with CTB

were transformed into *Agrobacterium* C58 strain (Department of Plant Genetics, VUB, Belgium). PCR approach was used to select positive clones. Specific oligonucleotides 5'LTB or 5' CTB and 3' cmyc were as following:

5' LTB: AAGTCGACATGGTGAAGGTGAAGTGC
5' CTB: AAGTCGACATGGTGAAGCTCAAG
3' cmyc: TCTAGATCAGAGTTCGTCTTTCAGATCCTCTTCTGAGATGAGTTTCTGTTCATCAGAATGAGCCAAAAG

PCR conditions were: 94°C, 5min, followed by 35 cycles: 94°C, 20s; 55°C, 30s; 72°C, 45s, and the final extension was 72°C, 10min.

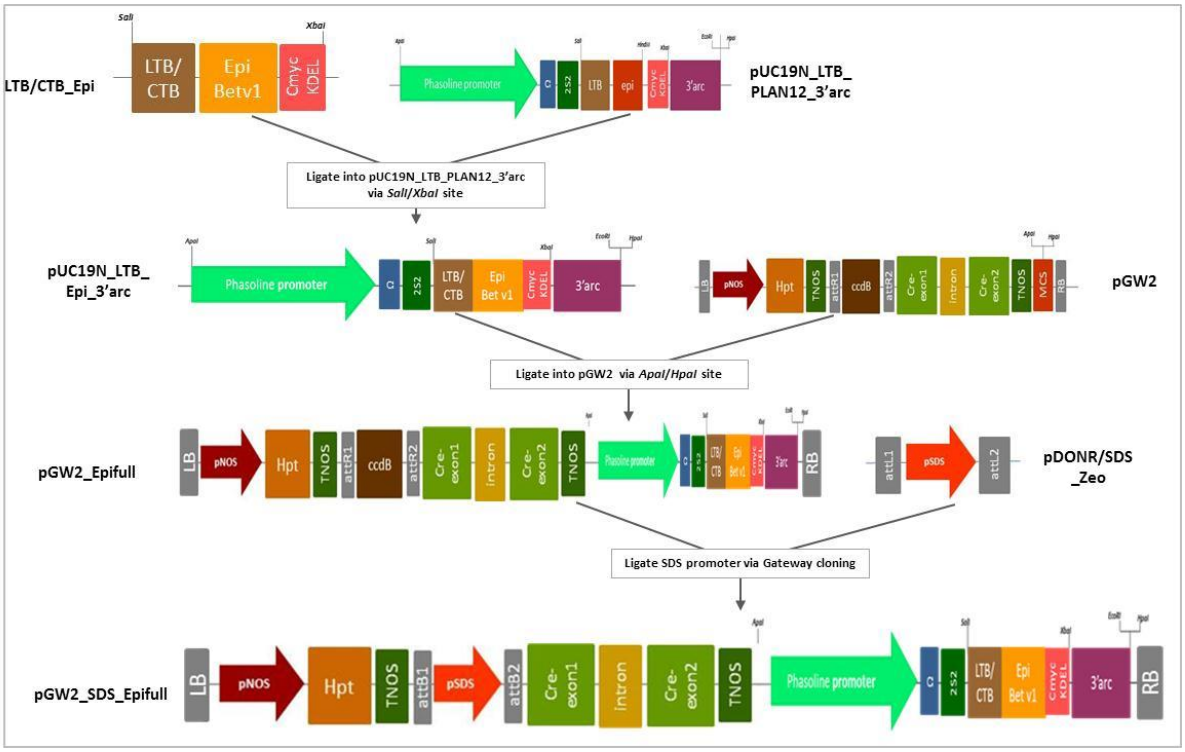

Fig. 1: Cloning strategy of pGW2_SDS_Epifull

2.4. Plant material, growing condition and transformation

Arabidopsis plants (A.thaliana, cv. Columbia) were cultivated in soil with 16h supplemental light followed by 8h darkness. The temperature regime followed by a day/night cycle with 25°C/20°C. About one-month-old flowering plants were used for Agrobacteium-mediated transformation via floral dip as described by (Clough SJ and Bent AF, 1998). In this method, transformation of female gametes is accomplished by simply dipping developing Arabidopsis inflorescences for a few seconds into a 5% sucrose solution containing 0.010.05% (vol/vol) Silwet L-77 and resuspended Agrobacterium cells carrying the genes to be transferred.

2.5. Selection of transformants

About three of four weeks after transformation, seeds were collected from treated Arabidopsis plants. To screen transformants, seeds of treated and wild type plants were surface sterilized as follows: 3 minutes in 70% ethanol, 30 minutes in 1% HClO followed by 6-8 times washing using distilled water. After one night treated at 4°C under dark condition, sterilized seeds were germinated using 16 h-light/8 h-dark intervals on K1 media (MS media containing 3% (w/v) sucrose, 0.48g/L MES pH 5.7) containing 0-50 mg/L hygromycine. After 2-3 weeks, hygromycine-resistant plants were transferred to the greenhouse and used for further analysis. Plants were cultivated in soil with 16 h of supplemental light followed by 8 h of darkness. Temperature was chosen to be 25°C/20°C according to the day/night cycles. Mature seeds were harvested from Arabidopsis plants after 10-12 weeks after pollination.

2.6. *RNA isolation and RT-PCR*

Total RNA was isolated from mature seeds of Arabidopsis plants TRIzol® reagents (Invitrogen, USA) with modified as following. For RNA isolation, 50mg seeds were homogenized in liquid air using motor and pestle and then 1mL of TRIzol® reagents were quickly added into each sample. After incubating at RT for 5min, each sample was added 200 µL chloroform, vortex for 15s, incubated at RT for 5min and centrifuged at 12,000 × g for 15 minutes at 4°C. Supernatant was transferred into new tubes, added 1/10 volume of 3M CH3COONa pH= 5.2 and 2.5V of 100% ethanol, mixed well and percipated at -20°C overnight. In the next day, pellet was collected by centrifuging at 12,000 × g for 15 minutes at 4°C. Pellet was washed twice by 500 µL of 3M CH3COONa pH= 5.2 and 500 µL of 70% ethanol. After drying at RT, pellet was dissolved by 50 µL of DEPC/H_20 and then two µg of total RNA were treated by RNase-free DNase for 30 min at 37°C, and the reaction was stopped by adding 1 µL of 25 mM EDTA followed by heat inactivation at 65°C for 10 min. RNA was reverse transcribed using the First Strand cDNA synthesis kit (Fermentas Life Science, Belgium) following the manufacturer´s instructions. One microliter of cDNA was applied for PCR using 5' LTB/CTB and 3'cmyc (part 2.3). RNA samples were normalized for initial variations in sample concentration using

the r18S controls. The r18S oligonucleotides comprising the sequence from nt401 to nt1220 (Unfried, Stocker and Gruendler, 1989) were as following:

5' *At*_r18S: ATCAACTTTCGATGGTAGG

3' *At*_r18S: CAGCCTTGCGACGACCATAC

Positive controls were 4 constructs above (1

2.7. IgG immunoblot

30 µg of total soluble proteins obtained from seeds of the different Arabidopsis lines or 5 µg of HA epitope-Cmyc protein (kindly provided by Dr. Tran Thanh Thu, VUB) were mixed with SDS containing sample buffer (Laemmli, 1970) and after heat denaturation total proteins were separated on 12.5% (v/v) SDS containing polyacrylamide gels under reducing conditions in a Mini-Protean 3 cell (BioRad, USA). Proteins were transferred onto Trans-Blot® Turbo™ Mini PVDF membranes (BioRad, USA) by tank blotting for 2x7min at 25 V (Trans-Blot® Turbo™ Transfer System, BioRad). After blocking in 3% BSA, 0,05% Tween 20, TBS pH 7,4 for 2 hours membranes were incubated overnight at 4°C with mouse antiserum raised against the myc protein (diluted 1: 700). Visualisation was carried out by BioRad alkaline phosphatase conjugate substrate Kit according to the manufacturer's instruction.

3. Result

3.1. Scanning for Bet v 1 epitopes

The birch pollen Bet v 1 is one of the five model allergens which have been well-characterized. Bet v is a small protein of 17kDa in molecular weight and comprising of 160 amino acids. From the nineties, the natural purified or recombinant Bet v 1 proteins were used to map the T-cell epitopes using T-cell clones from birch pollen allergic patients. Various linear T-cell epitopes within the Bet v 1 molecule were determined in individuals with birch pollen allergy as well as in nonallergic individuals (Ebner et al., 1993; Ebner et al., 1995). Most frequent epitopes were identified including the amino acid position: 75-92, 109-128 and 141-156. Later, in a study with 57 birch pollen allergy adults, Schmidt showed 61%; 40%; 28% of T cell clones from these donors reacted with Bet v $_{142-156,}$
Bet v 1 $_{112-123}$, Bet v 1 $_{94-111}$ respectively. The peptide from amino acid 142-151 in Bet v 1 molecule was identified as the dominant T-cell epitope (Schmidt et al., 2005). This was further confirmed using naturally processed peptides (Mutschlechner et al., 2010). Interestingly, the peptide Bet v $_{142-156}$ shares a high sequence homology with the C-terminal ends of Bet v 1- related food allergy. The comparison of Bet v $_{142-156}$ sequences to corresponding sequences from some Bet v 1-related food allergens showed the similarities ranging from 60-80%. This could explain the high-cross reactivity of birch pollen allergy to many plant-derived foods such as apples, carrots, tomatoes,…

Based on information of Bet v 1 epitopes, studies on 3D-crystal structure and modeling of Bet v 1 molecule together with computer-based algorithm predicted a number of peptides presenting the binding capacity to MHC molecules. Among these, two peptides Bet v 1 $_{141-156}$ and Bet v 1 $_{51-68}$ did not react with IgE Abs from sera from Bet v 1 allergic patients revealing that these peptides do not appear to contain epitopes recognized by Bet v 1-specific IgE (Nagato et al., 2007). Synthetic peptide of these two epitopes stimulated T cells, confirming the functional of them in Bet v 1 allergic

reactions. Therefore, the two epitopes Bet v 1 $_{141\text{-}156}$ and Bet v 1 $_{51\text{-}68}$ could be effective peptides for immunotherapy against birch pollen allergy.

Using the key word " birch pollen allergy+Bet v 1", 36 amino acid sequences representing pollen Bet v 1 was found out from the allergen database (www.allergen.org). Comparison the deduced amino acids sequences exhibited the high similarities between these isoforms ranging from 80% to 95%.

From the early study up to date, the Bet v 1 $_{141\text{-}156}$ was identified as the dominant T-cell epitope of Bet v 1. Additionally, the role of Bet v 1 $_{51\text{-}68}$ in stimulating T cells was proven. Therefore, in our study the epitope Bet v 1 $_{141\text{-}156}$ was expressed separately or linkage with epitope Bet v 1 $_{51\text{-}68}$ in transgenic seeds of *A.thaliana* plants. The amino acid sequences of these two epitopes are as follows.

Bet v 1 $_{141\text{-}156}$: **GETLLRAVESYLLAHS**

Bet v 1 $_{51\text{-}68}$: **PGTIKKISFPEGFPFKYV**

3.2. Optimization of Bet v 1 T-cell epitopes

Based on the codon usage in *A.thaliana*, the nucleotide sequences of Bet v 1 $_{141\text{-}156}$ and Bet v 1 $_{51\text{-}68}$ were optimized as shown in fig. 2.

```
Majority                GGAACTATTAAGAAGATCAGTTTTCCTGAAGGCTTCCCTTTCAAGTACGT
                                10        20        30        40        50
Bet v 1 epitopes op.seq GGAACTATTAAGAAGATCAGTTTTCCTGAAGGATTCCCTTTCAAGTACGT 50
Bet v 1 epitopes.seq    GGAACCATTAAGAAGATCAGCTTTCCCGAAGGCTTCCCTTTCAAGTACGT 50

Majority                TGGCGGCGGCGAGACTCTTTTGAGGGCTGTTGAGAGCTACCTTTTGGCTC
                                60        70        80        90        100
Bet v 1 epitopes op.seq TGGCGGCGGAGAGACTCTTTTGAGAGCTGTTGAAAGCTACCTTTTGGCTC 100
Bet v 1 epitopes.seq    GGGCGGCGGCGAGACACTTTTGAGGGCCGTTGAGAGCTACCTTTTGGCAC 100

Majority                ATTCTGAT

Bet v 1 epitopes op.seq ATTCTGAT                                          108
Bet v 1 epitopes.seq    ACTCCGAT                                          108
```

Fig 2: Comparison between natural and optimized Bet v 1 epitopes

The nucleotide sequences of these two epitopes were linked via Glycine linker GGC-GGC and fused to optimized nucleotide sequences of LTB/CTB via L-linker.

The role of heat-labile enterotoxin B subunit (LTB) in inducing mucosal immunization was proved. However, an efficient induction of oral tolerance by fusing cholera toxin B subunit with allergen-specific T-cell epitopes from Japanese cedar pollen was investigated (Takagi et al., 2008). Interestingly, the recombinant CTB-Bet v 1 (fusion with CTB and whole molecule of Bet v 1) was characterized as a candidate vaccine against allergic disease. The application of the fusion molecules via the mucosal/intranasal led to the reduction of Bet v 1-specific IgE resulting in the reduction of allergic symptoms (Bublin et al., 2007). In order to comparison the role of LTB/CTB fused with Bet v 1

epitopes as mucosal delivery system with immunosuppressive activity, the coding sequences of two proteins were optimized for higher expression in planta.
Figure 3 is the illustration of the recombinant LTB/CTB-epitopes.

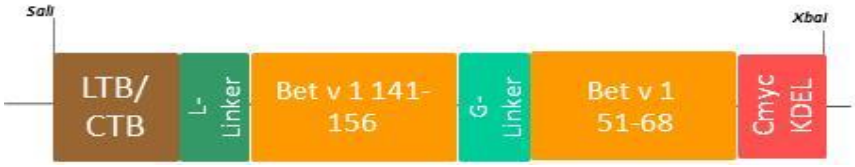

Fig 3: Scheme of Bet v 1 epitopes

The sequences of major components of recombinant LTB/CTB-epi are following:

Plant optimized synthetic LTB

ATG GTG AAG GTG AAG TGC TAT GTG CTC TTC ACT GCT CTC CTC AGC TCT CTT TGT GCT TAT GGA GCT CCA CAA TCC ATC ACT GAG CTT TGC TCT GAG TAC AGG AAC ACT CAG ATC TAC ACC ATC AAT GAC AAG ATC CTC TCT TAC ACT GAG AGC ATG GCT GGC AAG AGG GAG ATG GTG ATC ATC ACC TTC AAG TCA GGA GCC ACT TTC CAG GTG GAG GTT CCA GGC TCA CAA CAC ATC GAT TCC CAG AAG AAG GCC ATT GAG AGG ATG AAG GAC ACC TTG AGG ATC ACC TAC CTC ACT GAG ACC AAG ATT GAC AAG CTC TGT GTG TGG AAC AAC AAG ACT CCA AAC TCC ATT GCT GCC ATC AGC ATG GAG AAC

Plant optimized synthetic CTB

ATG GTG AAG CTC AAG TTC GGA GTG TTC TTC ACA GTT CTC CTC TCC TCC GCT TAC GCT CAT GGA ACA CCA CAG AAC ATT ACT GAT TTG TGC GCT GAG TAC CAC AAC ACA CAA ATT CAT ACT CTC AAC GAT AAG ATC TTC TCC TAC ACA GAG TCC CTC GCT GGA AAG AGG GAG ATG GCT ATC ATT ACT TTC AAG AAC GGA GCT ACT TTC CAA GTT GAG GTT CCA GGA AGC CAA CAT ATT GAT TCC CAG AAG AAG GCT ATT GAG AGG ATG AAG GAT ACC CTC AGG ATT GCT TAC CTC ACT GAG GCT AAG GTG GAG AAG TTG TGC GTT TGG AAC AAC AAG ACT CCA CAT GCT ATT GCT GCT ATT AGC ATG GCT AAC TAC

L-linker

TCA GAC CCA CAC GTG CCA AAC

Bet v 1 $_{141-156}$

GGC GAG ACA CTT TTG AGG GCC GTT GAG AGC TAC CTT TTG GCA CAC TCC GAT

G-linker

GGC GGC

Bet v 1 $_{51-68}$

GGA ACC ATT AAG AAG ATC AGC TTT CCC GAA GGC TTC CCT TTC AAG TAC GTG

C-myc tag

GAA CAG AAA CTC ATC TCA GAA GAG GAT CTG

KDEL

AAA GAC GAA CTC

3.3. Vector construction

Generation of vector backbone: pUC19N-LTB_PLAN12_3'arc

In previous Bilateral project between Belgium and Vietnam, the PLAN12 fragment containing the nucleotide sequences of phasoline seed-specific promoter, epitopes of HA protein from Influenza virus H5N1, omega leader, 2S2 signal peptide was artificial synthesized in pUC57 (Fig.4).

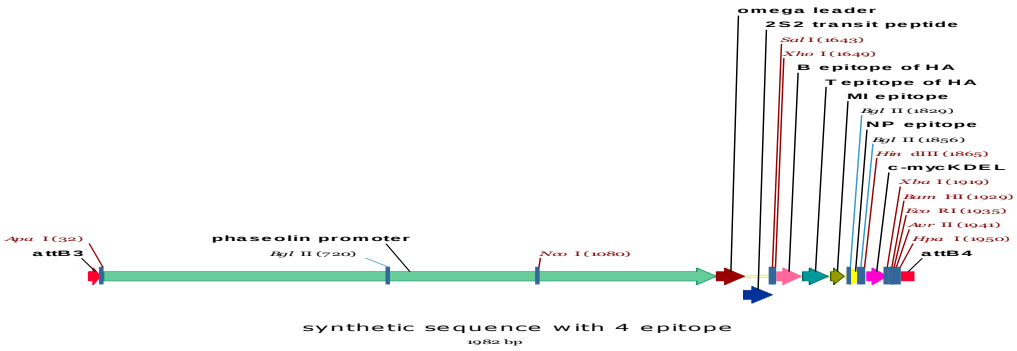

Fig. 4: The PLAN12 synthetic epitopes

In order to generate a binary plant transformation vector for high accumulation of recombinant protein in transformed cells, the LTB and 3'arc nucleotide sequences were planned to introduced in PLAN12 fragment. And then two epitopes of Bet v 1 were substituted HA epitopes via *XhoI/HindIII* sites. However, many of restriction sites in PLAN12 and pUC57are not unique. Therefore, the modification of PLAN12 synthetic was needed. To this end, we investigated in a modified pUC19 so called pUC19N with new Multi-cloning sites as following: *ApaI/SalI/XhoI/SacI/KpnI/StuI/NruI/ClaI/NotI/XmaI/PstI/StuI/SbfI/SpeI/HpaI.* The PLAN12 fragment was removed from pUC57 and introduced into pUC19N via *ApaI/HpaI* sites to obtain the pUC19N_PLAN12. As following, the LTB and 3'arc fragments were ligated into pUC19N_PLAN12 vector via *SalI/XhoI* and *EcoRI/XbaI* sites to generate pUC19N_LTB_PLAN12 and pUC19N_LTB_PLAN12_3'arc subsequently. The recombinant vectors were confirmed via vector digestion with appropriate restriction enzyme. The final vector was designated as pUC19N_LTB_PLAN12_3'arc (Fig. 3)

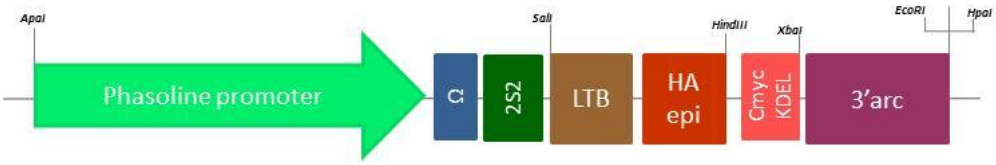

Fig 5: Illustration of pUC19N_LTB_PLAN12_3'arc

Generation of pGW2-EpifullSDS

Followed the cloning strategy (fig. 1), the *SalI/XhoI* LTB/CTB-epi fragments were inserted into pUC19N_LTB_PLAN19_3'arc via *SalI/XbaI* sites and then the LTB/CTB-epi-3'arc fragments will be introduced into pGW2 vectors. The SDS promoter will be ligated to demonstrate the final destination vector. The final vector is under constructions. However finally, we will use four following vector for plant transformation:

- pGW2_LTB/2Epi_SDS containing two epitopes Bet v 1 $_{141\text{-}156}$ and Bet v 1 $_{51\text{-}68}$ fused with LTB
- pGW2_LTB/1Epi_SDS containing epitope Bet v 1 $_{141\text{-}156}$ fused with LTB

- pGW2_CTB/2Epi_SDS containing two epitopes Bet v 1 $_{141-156}$ and Bet v 1 $_{51-68}$ fused with CTB
- pGW2_CTB/1Epi_SDS containing two epitopes Bet v 1 $_{141-156}$ fused with CTB

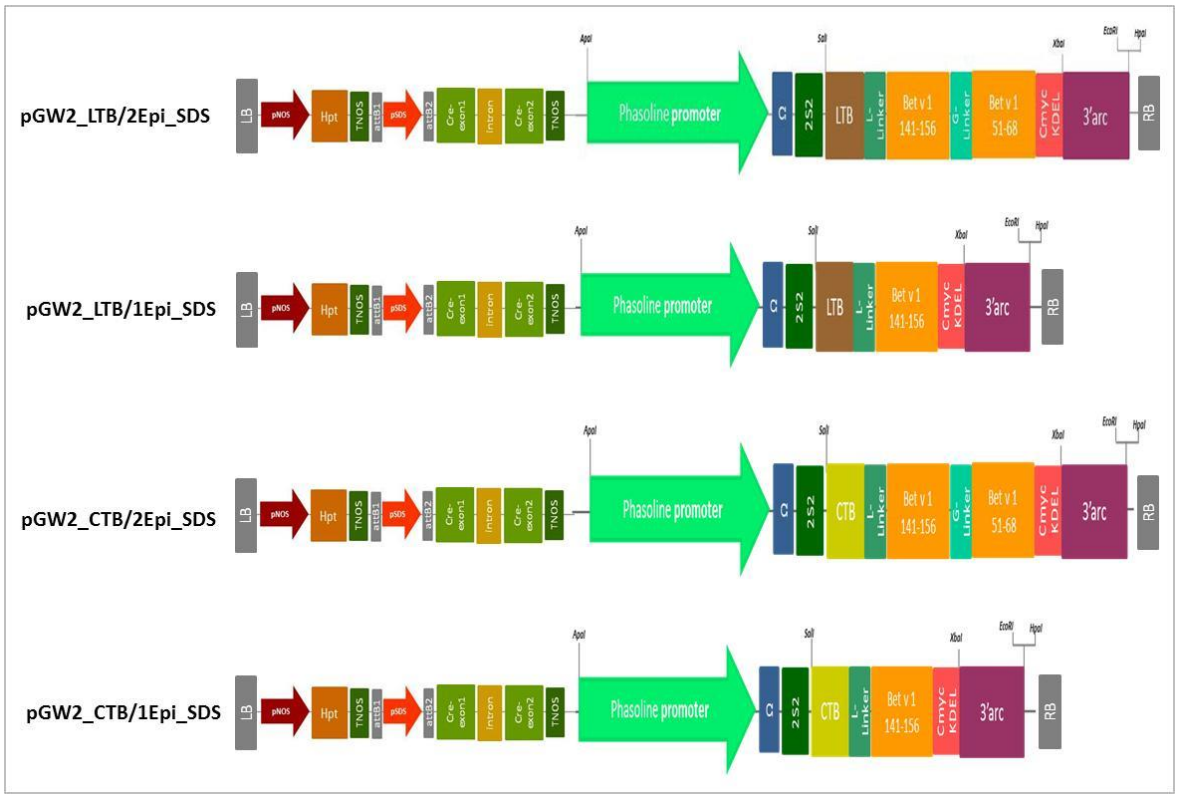

Fig. 6: Illustration of 4 binary vectors using for A.thaliana transformation

3.4. Generation of Bet v 1 T-cell epitope-expressing plants

To generate Arabidopsis seeds expressing the T-cell epitopes of birch pollen allergen Bet v 1, the codon of these epitopes were optimized and cloned in frame into a binary vector. To enhance the mucosal immunization and the expression, the coding sequence of LTB/CTB and the untranslated region 3'arc were subsequently ligated into binary vectors. The final binary constructs were transformed into Arabidopsis plants by *Agrobacterium*-mediated gene transfer via floral dip in separate experiments for each construct, and then screening of transformants by the selection of hygromycin-resistant plants.

Transformation of plant binary vectors into Agrobacterium

All of constructed vectors were transformed into Agrobacterium C58 strain. After transformation, plasmids were isolated from *Agrobacterium* colonies and applied as DNA probes for PCR. As shown in Fig. 7, the 450bp and 500 bp-fragments were amplified *Agrobacterium* clone 2 and *Agrobacterium* clone 3 using the specific oligo nucleotides 5' CTB/3'cmyc, respectively. The size of these fragments were similar to estimated size of CTB-1epi and CTB-2epi in plasmid pGW2_CTB/1Epi_SDS and pGW2_CTB/2Epi_SDS, respectively confirming the presence of these constructs in Agrobacterium clone 2 and Agrobacterium clone 3. PCR amplification using the 5' LTB/3'cmyc oligonucleotides exhibited similar results revealing the two constructs pGW2_LTB/2Epi_SDS and

pGW2_LTB/1Epi_SDS were transformed successfully into *Agrobacterium*. These positive clones were stored at -80^{o}C and used for plant transformation.

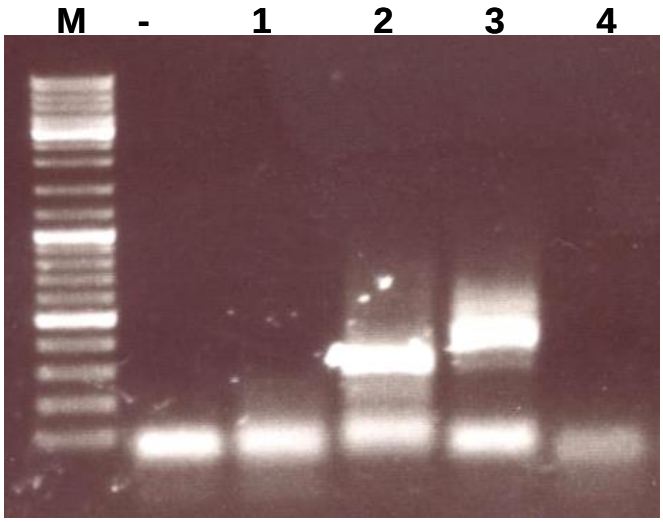

Fig 7: PCR amplification from plasmids isolated from Agrobacterium colonies
M: 1kb marker (Fermentas); (-): negative control; plasmids isolated from Agrobacterium colonies transformed with (1),(2): pGW2_CTB/1Epi_SDS; (3),(4): pGW2_CTB/2Epi_SDS

Screening of transformants

To allow selection of transgenic plants, the *hyg* gene coding for hygromycine resistance has been included in all transformation vectors. Therefore, transformants can be screened via hygromycine resistance. Seeds of Agrobacterium-treated plants were germinated on selective medium and hygromycin resistant plants were selected for further analysis. The concentration of hygromycine was 15 and 25mg/L as described by previous papers.

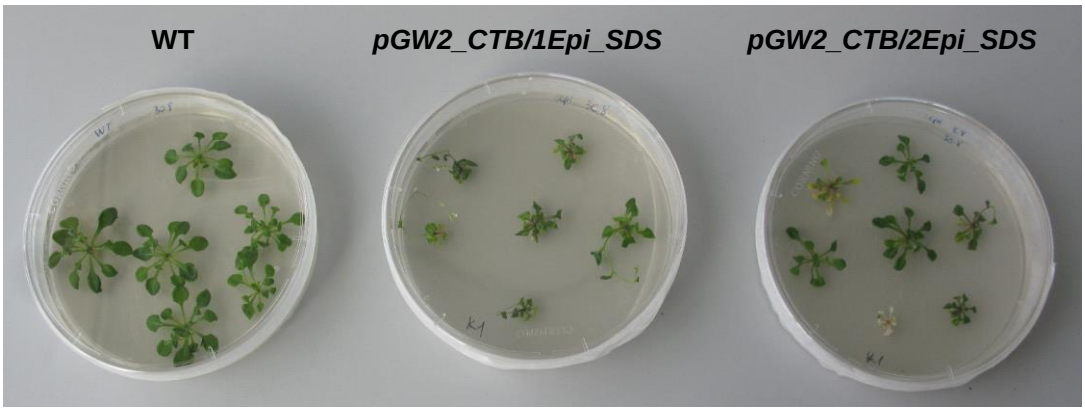

Fig 8: Arabidopsis plants after four weeks in selective medium

The germination and development of treated or wild-type seeds were observed from one to four weeks after. Following week by week, we observed that all types of seeds can germinate similarly in both K1 free and K1 containing hygromycine at all tested concentration. The difference

between treated and wild-type seeds was in the further developmental periods. Treated and wild type seeds exhibited no developmental difference at concentration of 15 and 25mg/L hygromycine. The concentration was shifted to 30 and 50mg/L. At concentration of 30mg/L, most of treated seeds still can germinate and developed full leaves while 70-80% of wild type seeds can. Most of treated seeds can germinated at 50mg/L hygromycine but 5-10% plants can grow and develop greenish leaves. The concentration of 50mg/L hygromycine was used as selection threshold in our further experiments.

Generation of T0 transgenic plants in green house

After three weeks on selective medium, resistance plants were re-cultured in K1 free medium for further week and then transferred to soil in greenhouse. At the moment, transgenic plants are flowering. No phenotypic changes between wild type and transgenic plants observed, suggesting that Bet v 1 epitopes are not toxic for Arabidopsis plants and this plant can be used as *"farmer"* for the production of Bet v 1 epitopes.

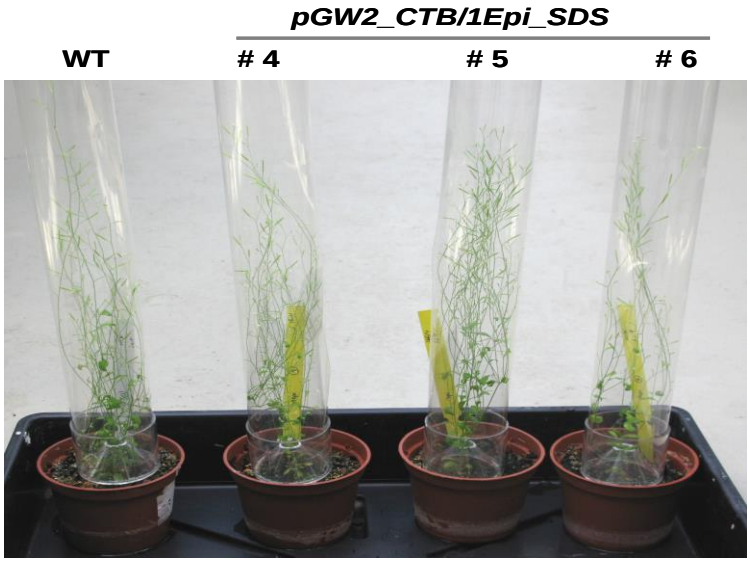

Fig 9: Illustration of several transgenic lines in the greenhouse

3.5. Screening Bet v 1 expressing plants

In order to produce the epitopes of birch pollen Bet v 1 in plant system, the epitope Bet v 1 $_{141\text{-}156}$ was clone separately or in conjugated with another Bet v 1 $_{51\text{-}68}$ epitope into plant expression vectors. To enhance the mucosal immunization and the expression, the coding sequence of LTB/CTB and the untranslated region 3'arc were subsequently ligated into binary vectors. Additionally, the Cmyc tag was fused in frame with the two epitopes and the final constructs were transformed into *Arabidopsis* plants via *Agrobacteria*-mediated transformation. After screening in hygromycine selection medium, ca. 30 plants of each construct were selected and transferred to green house for seed collection. A total of more than 100 different T1 transgenic plants were screened for expression of Bet v 1 epitopes in seed extracts. Protein extracts were separated on SDS-PAGE and blotted onto PVDF membranes. For immunodetection of Bet v 1 epitope-Cmyc fusion, the mouse polyclonal antibody raised against cmyc was used. Fig 10 is the illustration of some immunoblots from 11

different transgenic lines of more than 100 lines tested. Fig. 10 A showed the immunoblot of 5 different hygromicine-resistant pGW2_CTB/1epi_SDS lines (Line #4, 5, 6, 18, 20) and Fig. 10 B showed the immunoblot of 6 different hygromicine-resistant pGW2_CTB/2epi_SDS lines (Line #1, 2, 12, 17, 19, 31). The Cmyc antiserum detected strongly an 50 kDa protein corresponding to the HA epitope-Cmyc protein in 5 µg total seed protein loaded. In opposite, no specific bands were observed in seed extracts from nontransformed plants. Unfortunately, similar results were obtained in all transgenic lines tested even when the concentration of total protein was increased up to 50 µg which is ten times higher than positive control. Cmyc antiserum did not detect any protein bands from seed extracts of T1 transgenic lines from all four constructs. This indicates that the expression level of Bet v 1 epitopes was under detectable limits.

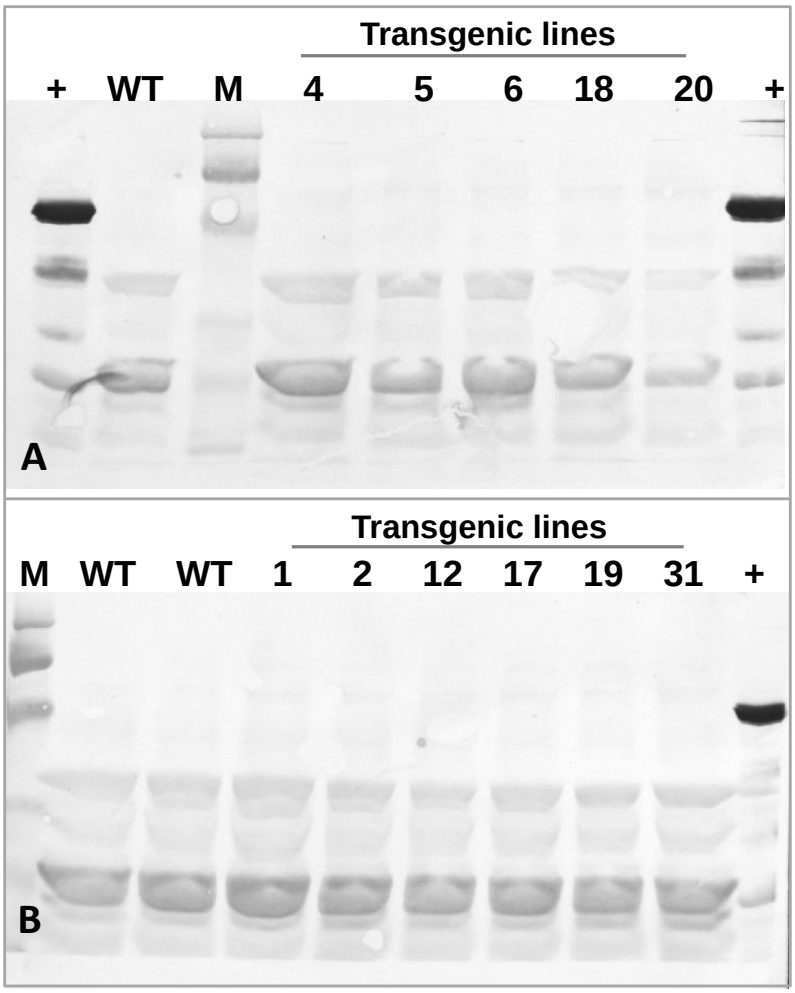

Fig. 10: Western blot analysis of transgenic plants.

Protein extracts were prepared from Arabidopsis seeds of different transgenic lines. Western blot was performed using a mouse-raised polyclonal anti-myc antiserum (dilution 1:700). (+): positive control: HA epitope-Cmyc protein; WT: non-transformed seeds; Fig. 10 A showed the immunoblot of 5 different hygromicine-resistant pGW2_CTB/1epi_SDS lines (Line #4, 5, 6, 18, 20) and Fig. 10 B showed the immunoblot of 6 different hygromicine-resistant pGW2_CTB/2epi_SDS lines (Line #1, 2, 12, 17, 19, 31).

Further, to confirm the presence of Bet v 1 epitope transcripts, total RNA was isolated from *Arabidopsis* seeds and treated with RNase-free DNase to remove all DNA contamination. Two microgram of total RNA from wild type and transgenic plants were reverse-transcribed into cDNA

and Bet v 1 epitope transcripts were amplified using 5'LTB/CTB and 3'cmyc oligonucleotides. Plasmids containing one or two Bet v 1 epitopes conjugated to LTB/CTB were used as positive controls, resulting in 400 and 500bp fragments, respectively (Fig. 11). These bands were not observed in Arabidopsis wild type seeds or in negative control of reactions in which water was used instead of cDNA. As shown in Fig. 11A, the 5'CTB and 3'cmyc oligonucleotides successfully amplified the 400bp fragment from cDNA isolated from seeds of five different pGW2_CTB/1epi_SDS lines (line 4, 5, 6, 18, 20). Similarly, the 500bp fragment was obtained in pGW2_CTB/2epi_SDS lines (Line #1, 2, 12, 17, 19). These results revealed the presence of Bet v 1 epitope transcripts in Hygromycine resistant lines.

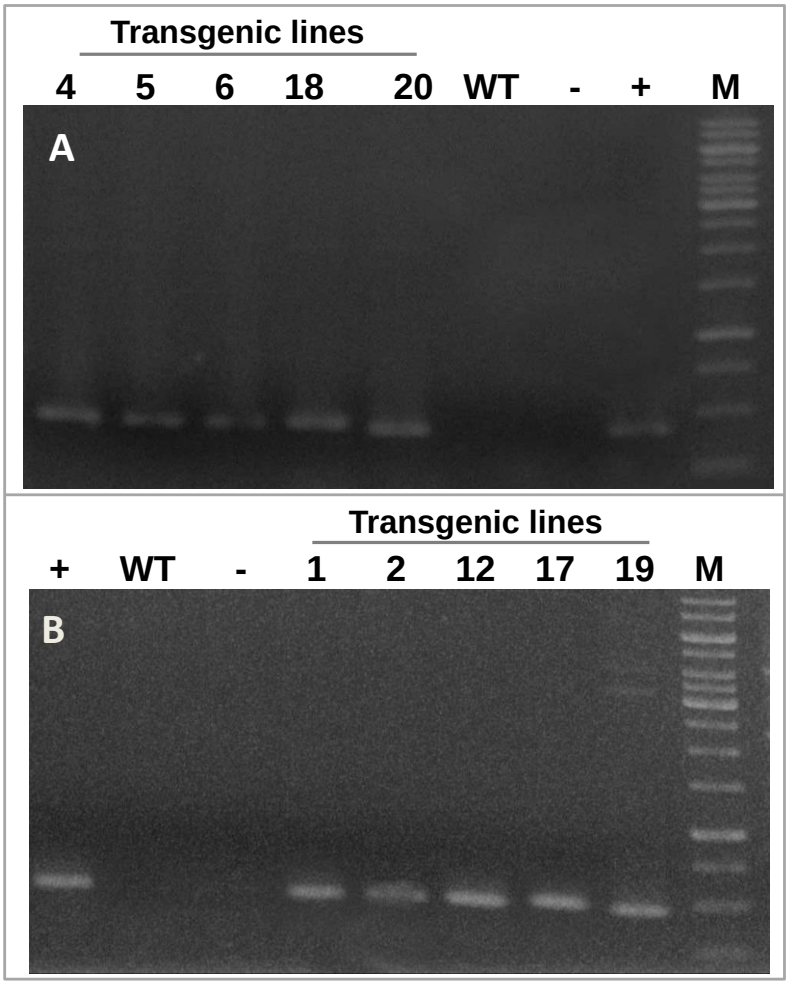

Fig. 11: RT-PCR analysis of transgenic plants.

cDNA were prepared from Arabidopsis seeds of different transgenic lines. Fig. 10A: (+): positive control pGW2_CTB/1epi_SDS; WT: non-transformed seeds; (-): water control; # 4, 5, 6, 18, 20: 5 different hygromicine-resistant pGW2_CTB/1epi_SDS lines; Fig. 10B: (+): positive control pGW2_CTB/2epi_SDS; WT: non-transformed seeds; (-): water control; # 1, 2, 12, 17, 19: 5 different hygromicine-resistant pGW2_CTB/2epi_SDS lines

3.6. Discussion

In this study, we aim to expression two different T-cell epitopes (Bet v 1 $_{141-156}$ and Bet v 1 $_{51-68}$) of the major birch pollen Bet v 1 in Arabidopsis seeds by changing the codon usage and increasing the expression. To this end, the nucleotide sequences of Bet v 1 $_{141-156}$ and Bet v 1 $_{51-68}$ were optimized based on the codon usage in *A.thaliana*. On other hand, the untranslated region 3'arc was cloned after the stop codon of CTB/LBT-epitopes-cmyc sequences. Unfortunately, although the Bet v 1

epitope transcripts were detected in many hygromycine resistant lines, no Bet v 1 epitope proteins were expressed in *Arabidopsis* seeds at T1 generation. Low protein expression in transgenic seeds could be caused by some reasons: (1) the Bet v 1 epitope transcripts were poorly recognized by the translation machinery thus the proteins were not translated; (2) the protein were translated but unstable in ER or (3) proteins were partly degraded especially the cmyc tag thus the antibodies raised against cmyc did not recognize the whole molecules. The stable of recombinant Bet v 1 epitopes were further analyzed by isolating protein via different protein extraction buffers containing protease inhibitor to prevent the protein degradation. We used three buffers as following:

- Lamli buffer containing Protease cocktail inhibitor (Sigma, USA) 1/10 (v/v)
- Lamli buffer containing 1mM PMSF (Sigma, USA)

30 µg of total soluble proteins were loaded into each lane of a 12.5% SDS-PAGE, blotted into PVDF membrane and incubated with a mouse antibody raised against myc tag. The additional of these protease inhibitors did not exhibit any significant changes in the components of protein bands after incubating of cmyc antibodies. Neither protein extracted in Lamli buffer containing PMSF nor protein extracted in Lamli buffer containing protease cocktail inhibitor formed any specific bands with cmyc antibody.

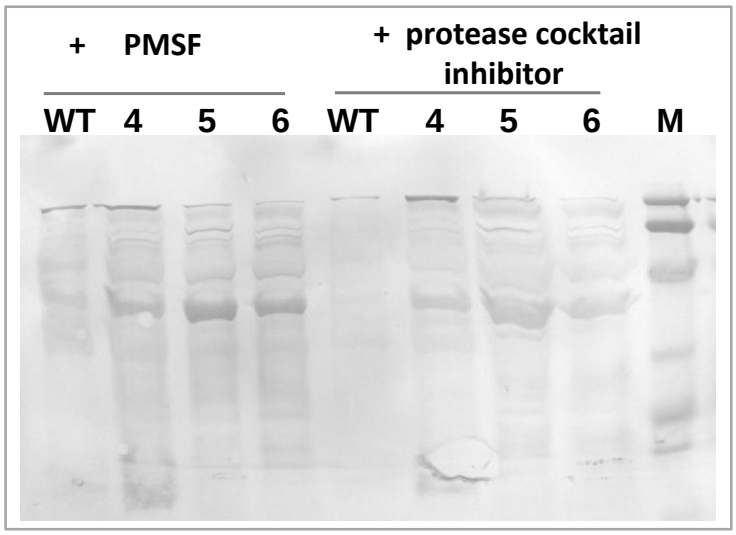

Fig. 12: WB analysis of Bet v 1 protein isolated in different extraction buffer

Protein extracts were prepared from Arabidopsis seeds using two different extraction buffer in which: ***+ PMSF*** *is Lamli buffer containing 1mM PMSF and* ***+ Protease cocktail inhibitor*** *is Lamli buffer containing Protease cocktail inhibitor 1/10 (v/v). Western blot was performed using a mouse-raised polyclonal anti-myc antiserum (dilution 1:700). Using protein extracts from 3 different hygromicine-resistant pGW2_CTB/1epi_SDS lines (Line # 4, 5, 6)*

Increase the expression level of recombinant protein is one of major tasks for molecular farming. In our study, the Bet v 1 epitopes were under detectable levels using cmyc antibody. Biemelt et al., (2003) improved the L1 protein expression in tobacco and potato plants by using the Ω translational enhancers of the tobacco mosaic virus (U1). The L1 accumalated increased from 0.5% to 2%. Following this strategy, this enhancer can be used in further studies.

4. Conclusion

Within the project, two major T-cell epitopes of Bet v 1 (Bet v 1 $_{141-156}$ and Bet v 1 $_{51-68}$) were identified. These epitopes were cloned via SOEing PCR resulting four different vectors. Via Agrobacterium-mediated transformation, these vectors transformed into *Arabidopsis* plants. The presence of these constructs in Arabidopsis seeds were confirmed via RT-PCR. However, no protein accumulation was revealed. Further study could focus on the modification of binary vectors to improve the expression levels.

Reference

Ahmad et al. Role of transgenic plants in agriculture and Biofarming. Biotechnol Adv. 30(3):524-40.

Basaran and Rodríguez-Cerezo (2008) Plant molecular farming: opportunities and challenges Crit Rev Biotechnol. 2008;28(3):153-72

Biemelt S, Sonnewald U, Galmbacher P, Willmitzer L, Müller M (2003) Production of human papillomavirus type 16 virus-like particles in transgenic plants. J Virol. 77: 9211-9220.

Bublin et al. (2007) Use of a genetic cholera toxin B subunit/allergen fusion molecule as mucosal delivery system with immunosuppressive activity against Th2 immune responses. Vaccine 25(50):8395-404

Clough SJ and Bent AF (1998): Floral dip: a simplified method for Agrobacterium mediated transformation of Arabidopsis thaliana. Plant J 16: 735-43

Ebner et al. (1993) Identification of multiple T cell epitopes on Bet v 1, the major birch pollen allergen, using specific T cell clones and overlapping peptides. *Journal of Immunology* 150: 1047-1054

Ebner et al. (1995) Non allergic individuals recognize the same T cell epitopes of Bet v 1, the major birch pollen allergen, as atopic patients *Journal of Immunology* 154: 1932-1940

Fischer et al. (2004) Plant-based production of biopharmaceuticals. *Curr Opinion in Plant Biol* 7: 152-158

Groenlund and Gafvelin (2010) Recombinant Bet v 1 vaccine for treatment of allergy to birch pollen. Human vaccine 6 (12): 970-977

Jarolim et al. (1989) Monoclonal antibodies against birch pollen allergens: characterization by immunoblotting and use for single-step affinity purification of the major allergen Bet v 1. *Int Arch Allergy Appl Immunol.* 90(1):54-60.

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227(5259), 680-685.

Lamphear et al. (2002) Delivery of subunit vaccines in maize seed. *J Controlled* release 85: 169-180

Larché (2006) Immunoregulation by targeting T cells in the treatment of allergy and asthma. *Cur Opin Immunol* 18: 745-50

Larché (2006) Peptide immunotherapy. *Immunol Allergy Clin North Am.* 26(2): 321-32

Ma et al. (2005) Plant-derived pharmaceuticals – the way forward. *Trends Plant Sci* 10: 580-585

Meno (2011) Allergen structures and epitopes. *Allergy* 66 (Suppl.95): 19-21

Mishra et al. (2008) Edible vaccine: a new approach to oral immunization Indian Journal of Biotechnology Vol 7: 283-294

Möbs et al. (2011) Birch pollen immunotherapy leads to the differential induction of regular T cells and delayed T helper cell immune deviation *Journal of Immunology 184: 2194-2203*

Mutschlechner et al. (2010) Naturally processed T cell activating peptides of the major birch pollen allergen. J *Allergy Clin Immunol.* 125(3):711-718

Nagato et al. (2007) Fuctional analysis of birch pollen allergen Bet v 1-specific regulator T cells. *Journal of Immunology* 178(2):1189-1198.

Rashid et al. (2011) Pollen-food syndrome is related to Bet v 1/PR-10 protein sensitisation, but not all patients have spring rhinitis. *Allergy* 66(10):1391-1392

Roitt I, Brostoff J and Male D (1993): Immunology, 3rd Edition. Mosby-Year Book Europe Ltd., England

Schmid et al. (2005) Bet v 1 142-156 is the dominant T-cell epitope of the major birch pollen allergen and important for cross-reactivity with Bet v 1-related food allergens. *J Allergy Clin Immunol.* 116(1):213-219

Streatfield SJ and Howard JA (2003) Plant-based vaccines. Intern J Parasitology 33: 479-493

Swoboda et al. (1995) Isoforms of Bet v 1, the major birch pollen allergen, analyzed by liquid chromatography, mass spectrometry and cDNA cloning. J Bio Chemistry 270: 2607-2613
Takagi et al. (2008) Efficient induction of oral tolerance by fusing cholera toxin B subunit with allergen-specific T-cell epitopes accumulated in rice seed. Vaccine 26: 6027-6030
Unfried, Stocker and Gruendler (1989) Nucleotide sequence of the 18S rRNA gene from Arabidopsis thaliana ColO Nucleic Acids Research 17 (18): 7513
Walmsley and Arntzen (2003) Plant cell factories and muscosal vaccines. *Curr Opinion in Biotech* 14: 145-50

SUMMARY

Type I hypersensitivity is the most common allergic reactions and mediated by the over-production of allergen-specific IgE antibodies in allergic individuals after allergen exposure. Based on this mechanism of IgE-mediated allergic reactions, it was believed that T cell epitopes posses an important role in immune deviation (Larché M, 2006; Larché M, 2006). Therefore, immunotherapy using T-cell epitopes could be an easy-established and effective strategy to induce adaptive immune response against allergy. Allergy to birch (*Betula pendula, syn. B. verrucosa*) is one of the most common pollinosis in Europe and prevalence of birch pollen sensitization has continuously increased year by year. Comprehensive studies on birch pollen allergy identified two birch pollen allergens in which Bet v 1 is considered as the major allergen of birch pollen allergy (Jarolim et al, 1989). In order to provide a proof of concept for using T-cell epitopes for immunotherapy against the inhaled pollen allergy, Bet v 1 was selected as a model allergens.

Within our study, two major T-cell epitopes including Bet v 1 $_{141-156}$ and Bet v 1 $_{51-68}$ were identified. Further, the nucleotide sequences of Bet v 1 $_{141-156}$ and Bet v 1 $_{51-68}$ were optimized based on the codon usage in *A.thaliana* to eliminate rare codons and improve protein expression levels. To enhance the mucosal immunization and the expression, the coding sequence of LTB/CTB were conjugated in frame to these epitopes. Besides, the untranslated region 3'arc were subsequently ligated into binary vectors to enhance the protein expression and stability. We constructed four different binary vectors containing one epitope (Bet v 1 $_{141-156}$) or two epitopes (Bet v 1 $_{141-156}$ and Bet v 1 $_{51-68}$) conjugated with LTB or CTB. A myc tag was also added in the C-terminal of recombinant protein for further analysis. To allow selection of transgenic plants, gene coding for Hygromycin resistance has been included in all transformation vectors. Transformants were preliminarily screened on MS medium containing 50mg/L hygromycin. *Ca.* 30 resistant plants of each construct were transferred to green house for further screening of Bet v 1 epitope expression in seeds at T1 generation. RT-PCR using 5'LTB/CTB and 3'cmyc oligonucleotides revealed the presence of Bet v 1 epitope transcripts in Hygromycine-resistant lines. However, the mouse antibody raised against cmyc did not detect any Bet v 1 epitope proteins accumulated in T1 seeds of all transgenic lines. The low level of protein expression could be explained by the poorly recognization of Bet v 1 epitope transcripts by the translation machinery or the cmyc fragment degradation thus the antibodies raised against cmyc did not recognize the whole molecules. Further study could focus on the modification of binary vectors to improve the stability and expression levels.

Keywords: *allergy, Bet v 1, T-cell epitopes, immunotherapy, protein expression, molecular farming*