Immunomodulation of Brassinosteroid Functions in Seeds of Arabidopsis thaliana (L.)

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List of abbreviations

ALP ..... alkaline phosphatase

ABA ..... abscisic acid

amp^r ..... ampicillin resistance

anti-Bl scFv..... anti-brassinolide single-chain Fv antibodies

BA 6-Benzyladenine

BAK1..... BRI1 associated receptor kinase1

Bl..... brassinolide

BRI1 ..... brassinosteroid-insensitive

BRs..... brassinosteroids

BSA ..... bovine serum albumin

c-myc..... c-myc tag sequence

CaMV35S ..... cauliflower mosaic virus 35S promoter

Cs..... castasterone

24-Cs 24-epicastasterone

E.coli..... *Escherichia coli*

EBL 24-epibrassinolide

ELISA..... enzyme-linked immunosorbent assay

ER..... endoplasmic reticulum

GA ..... gibberellic acid

GC-MS ..... gas chromatography-mass spectrometry

GFP..... green fluorescent protein

GST ..... glutathione S-transferase

HSPs ..... heat-shock proteins

JA ..... (3R,7R)-jasmonic acid

JAVII..... transgenic plants with anti-JA scFv targeted into the cytosol

KDEL ..... retention sequence

LEA ..... late embryogenic abundant

LeB4..... legumin B4 promoter

LeB4SP..... legumin B4 signal sequence

MS ..... Murashige-Skoog

NAA ..... 1-Naphtalene acetic acid

ori ..... origin of replication

PB..... protein body

PCR ..... polymerase chain reaction

RLKs ..... receptor-like protein kinases

poly A ..... polyadenylation signal

RP ..... ribosomal proteins

rpm ..... revolutions per minute

PSV ..... protein storage vacuole

scFv ..... single-chain variable fragment

sp ..... signal peptide

TAK16 ..... transgenic plants with anti-Bl A16 scFv expression in ER under control of LeB4 promoter

TBU16 ..... transgenic plants with anti-Bl A16 scFv expression in ER under control of USP promoter

TSP ..... total soluble protein

USP ..... unknown seed-specific promoter

WT ..... wild type

Contents

1. Introduction

1.1. The history of brassinosteroids

Brassinosteroids (BRs) have been generally accepted as steroidal plant hormones only recently. Since the discovery of brassinolide (Bl) (Figure 1), isolated from rape (*Brassica napus L.*) in 1979 by Grove et al. extensive studies have been undertaken worldwide on this notable substance. Diverse species of plants have been found to contain Bl. Their characteristic physiological effect on growth and development of plants as well as their potential abilities in agricultural applications have started to be examined. These compounds were grouped in a new class of plant hormones. Before the isolation of Bl, long discussion had been carried out, whether steroidal hormones exist in plants or not. In the year of 1979, when the isolation of Bl was reported, such doubts came to the end, because Bl has a steroidal structure and has apparent hormonal properties. After the Grove's report, castesterone (Cs) was isolated as the second BRs at the University of Tokyo (Yokota et al., 1982). Since then, a number of related steroidal compounds have been isolated from a variety of plant sources. To date, more then 40 free BRs and 4 BR conjugates have been found and fully characterized by spectrometric methods. Presumably, there is a number of yet unknown BRs and BR conjugates in plants. BRs have been found in at least 44 plant species. Some data suggest that BRs are more widely distributed than presently established. These findings suggest, that BRs are ubiquitously distributed in the plant kingdom.

At first, investigations were focused on describing the biosynthetical pathways of BRs and their counterparts in plants. Recently, the major part of the biosynthesis of Bl has been established (Fujioka and Sakurai, 1997). This hypothetical model is based on information from extensive step-wise feeding studies performed with radiolabelled precursors of Bl and analysis of their bioconversion products. In the last decade, attention concentrated also to the physiological properties of BRs (Sakurai and Fujioka, 1993; Adam et al., 1996, Fujioka and Sakurai, 1997; Sasse, 1997; Yokota, 1997). High interest in the function of BRs has been elicited through the strong responses of intact plants and explants observed upon application of exogenous BRs. They were considered as promising compounds for application in agriculture, because they showed different regulatory activities on growth and development in plants. Because BRs affect a broad range of biological activities, it has long been difficult to determine their functions in plant growth with a simple treatment.

Although the BRs are present in plant tissues, their importance for the regulation of plant growth and development has not been accepted as wide as for "classical" plant hormones such as auxins, gibberellins, cytokinins, jasmonates, ethylene and abscisic acid (Kende and Zeevaart, 1997). This situation changed dramatically with several reports on the identification and characterization of BR biosynthesis and signal transduction mutants (Clouse, 1996, 1997; Yokota, 1997). The phenotypes of the BR-deficient mutants presumed, that BRs are essential hormones and take part in light-regulated development.

A new attempt to identify BR-insensitive mutants of *Arabidopsis* resulted in the isolation of the dwarf mutant named *bri1* (Clouse et al., 1996; Szekeres et al., 1996). BRI1 was found to have strong homology to leucine-rich receptor kinases. Recent analysis of a range of BR biosynthesis and insensitivity mutants in *Arabidopsis* was decisive in discovering the physiological importance of BRs. Although we have a limited knowledge about mode of action of BRs, research is

moving rapidly along three convergent lines of analysis. Microchemical techniques are revealing the details of biosynthesis, distribution, and metabolism. The analysis of BR-deficient and BR-insensitive mutants aims to characterize the physiological roles of BRs in growth and development. The cloning of BR-regulated genes is providing insight into the molecular mechanisms of plant steroid hormone action. In future, the research of BRs will become increasingly important in understanding the plant growth regulation.

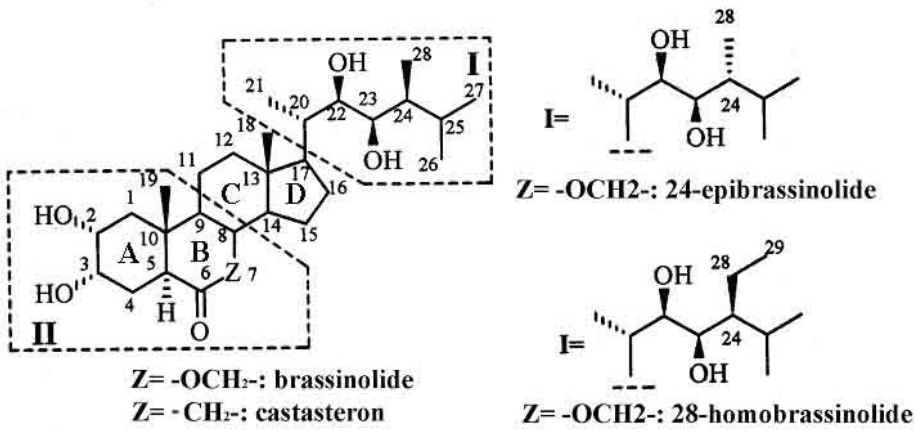

Figure 1. Brassinosteroids structure.

1.2. Natural occurrence of brassinosteroids in the plant kingdom

Among plant steroids only BRs are ubiquitously distributed throughout the plant kingdom. They play essential roles in modulating the growth and differentiation of cells at nanomolar to micromolar concentrations (Clouse and Sasse, 1998). All BRs are hydroxylated derivatives of cholestane and, given the possibilities of combinations of substructures in rings A and B and the side chain, the family has many more members then it is reported to date. The compounds can be classified as C_{27}, C_{28}, or C_{29} BRs, depending on the pattern of the side chain (Figure 1). BRs have been found in a wide range of plant species, in algae and pteridophytes, and three families of gymnosperms; in angiosperms, they have been shown to occur in 16 families of dicots, and 15 of monocots (Clouse and Sasse, 1998). They were detected in various plant parts such as pollen, seeds, leaves, roots, stems and flowers. Thus, BRs are widely distributed in the plant kingdom, and they are possibly biosynthesized in all parts of plant organs. Certainly they occur in shoots and seeds of the important experimental plant *Arabidopsis thaliana* (Fujioka et al., 1996, Schmidt et al., 1997). Levels of endogenous BRs vary among plant tissues. The highest measured concentration is about 10^{-1} nmol g^{-1} fresh weight (Bl in the pollen of *Brassica napus* and *Vicia faba*) and the lowest is about 10^{-7} nmol g^{-1} (homocastasteron in immature seeds and sheaths of Chinese cabbage, *Brassica campestris* var. *pekinensis,* Khripach et al., 2000). Pollen and immature seeds, the original sources of Bl, are containing the most of the hormones with ranges of 1-100ng x g^{-1} fw, wile shoots an leaves usually have lower amounts of 0,01-0,1 ng x g^{-1} fw (Takatsuto, 1994). The average steroid content in mature seeds of *Arabidopsis thaliana* is illustrated by recent data of Fujioka et al. (1998): brassinolide, $3{,}9x10^{-3}$ nmol g^{-1}; castasteron, $9{,}5x10^{-4}$ nmol g^{-1}; typhasterol, $3x10^{-3}$ nmol g^{-1}; 6-

deoxocastasterone, $3{,}5x10^{-3}$ nmol g^{-1}; 6-deoxotyphasterol, $2{,}1x10^{-3}$ nmol g^{-1}; 6-deoxoteasterone, $1{,}2x10^{-3}$ nmol g^{-1}. Bioassay results suggest that roots also contain BRs, however, possibly due to their low concentration in the tissue, they were not isolated yet (Clouse and Sasse, 1998).

1.3. Biosynthesis

Plant sterols have been extensively studied in the past years with a major focus on biosynthetic and biochemical aspects. Elucidation of BR biosynthesis and metabolism is fundamental to understand how plants regulate the endogenous level of active BR for their proper growth and development. Until recently, pathways of BR biosynthesis have remained unclear. Now Bl, the most important BR in plants, has been shown to be synthesized via two pathways from campesterol (Figure 2). Sterols are synthesized via the mevalonate pathway of isoprenoid metabolism. BRs must be converted from certain components of plant sterols, similar to animal and insect steroidal hormones which are derived from cholesterol. Among plant sterols, campesterol and its analogues are assumed to be the biosynthetic precursors of Bl, based on the identity of the side-chain skeleton. Early steps of Bl biosynthesis were investigated using feeding of cell cultures of *C. roseus* (Fujioka et al., 2000) with ^{2}H-labeled 6-oxocampestanol, 6-deoxocastasterone and 6α-hydroxycastasterone, and the metabolites were analyzed by gas chromatography-mass spectrometry (GC-MS). The cell cultures produced representatives of C_{28} BRs, such as Bl, Cs, typhasterol, testasterone and cathasterone (Choi et al., 1996, Fujioka et al., 1995). The levels of BRs in cell cultures of *C. roseus* were found to be comparable to those of BRs-rich plant tissues such as pollen and immature seeds. Using the cell culture system of *C. roseus*, biosynthetic studies of Bl were carried out by GC-MS analysis of the metabolites obtained from feeding labeled substrates.

Campesterol as the major plant sterol was found in cell cultures. To investigate the metabolites of this steroid, labeled campesterol was effectively prepared from labeled mevalonic acid. By feeding the cells with radioactive campesterol, campestanol, 6α-hydroxycampesterol, and 6-oxocampestanol were identified as metabolites. Subsequent feeding experiments with labeled campestanol and 6α- hydroxycampesterol revealed the biosynthetic sequence from campesterol to 6-oxocampestanol as shown in Figure 2 (Suzuki et al., 1995). This sequence constitutes an early part of the biosynthetic pathway of Bl, since the introduction of vicinal hydroxyls in the side chain of 6-oxocampestanol will yield teasterone. Therefore, a biosynthetic pathway for BRs via hydroxylations and epimerisation after the oxidation at C6 of the B-ring was suggested. This pathway leading to Bl was proposed as an early C6-oxidation pathway. Transformation of 6-oxocampestanol to teasterone was described using chemically synthesized specimens of possible intermediates as probes. As a result, 22α-hydroxy-6-oxocampestanol was identified and named cathasterone (Fujioka et al., 1995) In the same work cathasterone was identified as is the direct precursor of teasterone. By feeding experiments with deuterium-labeled teasterone, it was shown to be converted to typhasterol. This epimerization would occur via oxidation to the 3-oxo-form. 3-Dehydrotesterone was shown to be reduced to typhasterol as major metabolite and to teasterone as minor one (Suzuki, 1995). The conversion of typhasterol to Cs and Bl was demonstrated by feeding with deuterium-labeled substrate. Typhasterol was predominantly converted to castasterone, while minor epimerization to teasterone was observed. Convertion of Cs to Bl was at first shown by feeding with deuterium-labeled castasterone (Suzuki et al., 1993), and later conclusively demonstrated by feeding with tritium-labeled Cs

(Yokota et al., 1990). Thus, the biosynthesis of Bl via the early C6-oxidation pathway was described as shown in Figure 3.

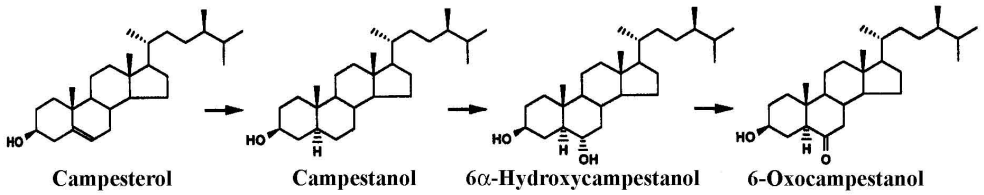

Figure 2. Biosynthetic pathway from campesterol to 6-oxocampestanol

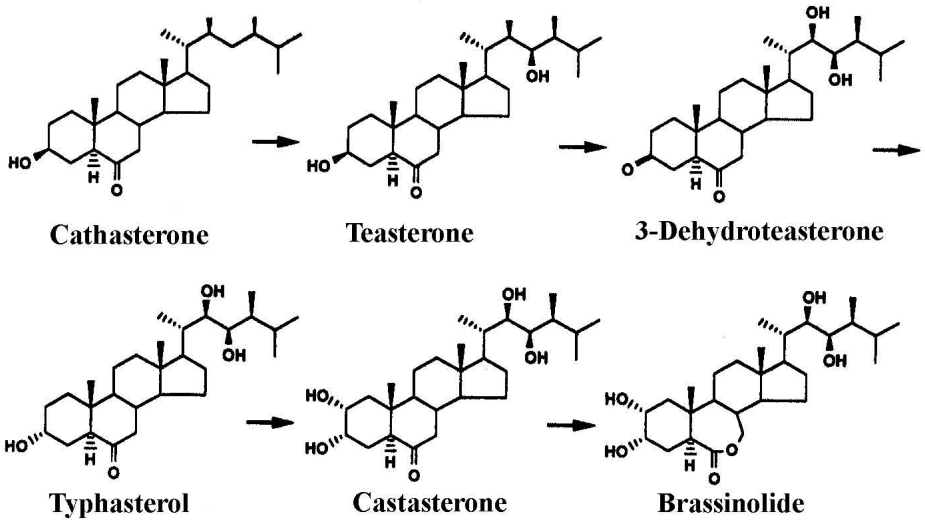

Figure 3. Biosynthesis of Bl via early C6-oxidation pathway.

Among natural BRs, attention was not drawn to the 6-deoxo BRs such as to 6-deoxocastasterone. They were considered to be dead-end in the pathway and not to be converted to active BR. However, reports showing the natural occurrence of 6-deoxo BRs in many plants (Abe et al., 1994; Yokota et al., 1994) have allowed their involvement in the biosynthetic pathway to be reevaluated. Feeding experiments with deuterium-labeled substrate revealed the conversion of 6-deoxoteasterone to 6-deoxotyphasterol. Similarly, 3-dehydro-6-deoxoteasterone was shown to be converted to 6-deoxotyphasterol. Thus, 6-deoxoteasterone is epimerized to 6-deoxotyphasterol via the 3-oxo form (3-dehydro-6-deoxoteasterone), similar to the convension of teasterone to typhasterol (Choi et al., 1997). Moreover, 6-deoxocastasterone was found to be converted to Cs and Bl (Choi et al., 1996). Therefore, the existence of an alternative Bl biosynthetic pathway via late C6-oxidation was demonstrated as shown in Figure 4. Both the early and the late C6-oxidation pathways operate in cell culture of *C.roseus*. The co-occurrence of BRs, belonging to the pathways in the same plant species indicates that these pathways could be ubiquitous in plants (Fujioka and Sakurai, 1997).

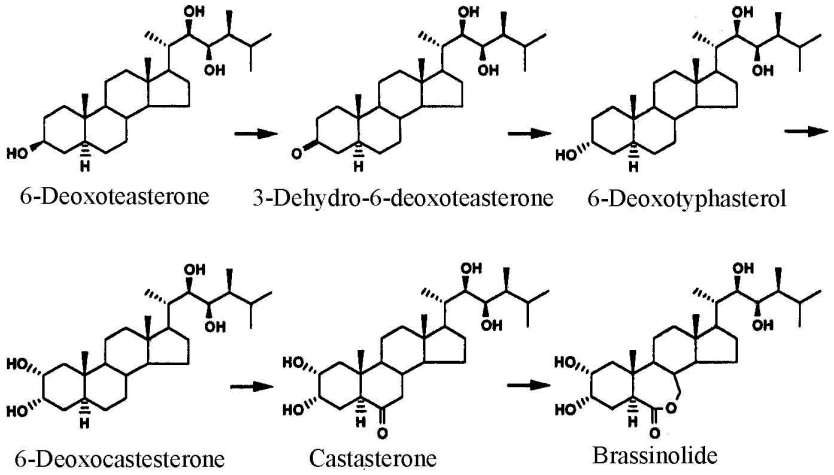

Figure 4. Biosynthesis of Bl via late C6-oxidation pathway.

1.4. Physiological action of brassinosteroids

In 1970 Mitchell et al. reported about the discovery of new hormones, which they called "brassins". They were inducing very marked elongation of both second and third internodes of the intact plants of *Brassica napus* (Mitchell et al., 1970). Further, the response to "brassins" was histologically different from the response induced by gibberellic acid (GA). Using chromatographic methods, the product, termed "brassins" was isolated from the pollen of *Brassica napus*. They were found to be a family of plant hormones that appear to have a glyceride structure. Later reports claimed a wide range of physiological effects of Bl in plants: elongation, bending, cell division, reproductive and vascular development, membrane polarization and proton pumping and modulation of stress (Sakurai et al., 1999). In addition to their essential role in plant development, BRs have the ability to protect from various environmental stresses. Treatment of *B. napus* with 24-epibrassinolide (EBL) leads to a significant increase in their basic thermotolerance, and results in higher accumulation of four major classes of heat-shock proteins (HSPs) as compared to untreated seedlings (Dhaubhadel et al., 2002). Kalinich et al. (1985) have shown that Bl treatment enhanced the activities of RNA and DNA polymerases as well as the levels of RNA, DNA, and protein and suggested that Bl responses are dependent on nucleic acid and protein synthesis. Exogenously applied Bl increases graviotropic curvature in maize (*Zea mays*) primary roots (Kim at al., 2000). Morillon et al. (2001) hypothesized about the role of Bl in the modification of the water-transport properties of cell membranes. Application of Bl to the stigmas of castrated flower buds is inducing parthenogenetic haploid seed germination in *A. thaliana* and *B. napus* (Kitani, 1994). *BRs* also interact with environmental signals and can affect insect and fungal development. A study of growth of monocots and dicots showed a promotive effect of a Bl isomer on photosynthetic capacity and biomass production in their primary developmental stages (Braun and Wild, 1984). In mustard, fresh weights of the shoots increased, and both elongation and radial growth were stimulated. An increase of 40-50% in CO_2 fixation *in vivo* in wheat has

been measured. The levels of soluble proteins and reducing sugars also increased, but chlorophyll levels were only slightly affected. On hydroponically grown *Arabidopsis* plants, with application of BR to the roots, profound effect on petiole elongation and upward bending of the leaf was detected (Atreca and Arteca, 2001). Elongation was promoted in young stem tissues of many plants after treatment with moderate doses of BR. This could be due to either higher endogenous auxin levels in the tissue or higher sensitivity of young tissue to BR. Evidence for BR-auxin synergism comes from the data of auxin-induced ethylene production in etiolated mung bean hypocotyls segments (Arteca et al., 1985). Several authors proposed that BR-induced effects might be mediated via auxin, that BR treatment might alter the level of endogenous hormones, or that BR might enhance tissue sensitivity to auxin (Mandava, 1988). This synergism, however, occurs only when the tissue is pretreated with BR and then exposed to auxin. When the order is reverse, there is no effect. Stimulatory effects of BRs on elongation are among the most documented physiological effects.

Steber and McCount (2001) have shown, that BR stimulates germination in *Arabidopsis,* proposing, that BRs are needed to antagonize seed dormancy and stimulate germination. Leubner-Metzger (2001), observed that Bl in seed germination of *Nicotiana tabacum* did not release photodormancy of dark-imbibed photodormant seeds in contrast to the GA, and suggested that Bl but not GA, accelerated endosperm rupture of tobacco seeds imbibed in the light. His results indicated that BRs and GAs promote tobacco seed germination by distinct signal transduction pathways and distinct mechanisms. Gibberellins and light seem to act in a common pathway to release photodormancy, whereas BRs do not release photodormancy. BRs seem to promote seed germination by directly enhancing the growth potential.

In addition, a steroid hormone, brassinolide, also plays a role in light-regulated development and gene expression in Arabidopsis (Chory et al., 1996; Szekeres et al., 1996; Li et al., 1996; Nagata et al., 2000).

Examination of the phenotype of BR-deficient and insensitive mutants confirmed, that many of the effects observed by exogenous application of BRs to bioassay systems in fact do occur *in planta*.

BRs were considered promising compounds for application in agriculture, because they showed various kind of regulatory activity on growth and development in plants (Table 1).

Table 1. Physiological effects of brassinosteroids in plants. According to Khripach et al. (2000)

Cell level	Whole plant level
Stimulation of elongation and fission	Growth promotion
Effect of hormonal balance	Increase in the success of fertilization
Effect on enzyme activity; H^+-pump activation	Shortening the period of vegetative growth
Activation of protein and nucleic acid synthesis	Size and quantity of fruits increase
Effect on the protein spectrum and on the amino acid composition of proteins	Effect on the content of nutritive components and fruit quality
Effect on the fatty acids composition and on the properties of membranes	Increased resistance to unfavourable environmental factors, stress and diseases
Enhancement of the photosynthesis capacity and of translocation of products	Crop yield increase

1.5. Molecular genetics of brassinosteroid action

1.5.1. Brassinosteroid signal transduction

Until very recently, almost all BR signal transduction research focused to Brassinosteroid Insensitive 1 (BRI1*)*, a single genetic locus in *Arabidopsis* encoding a leucine-rich repeat receptor kinase (Clouse et al., 1996). The putative model of BR signal transduction suggests a pathway that combines some of the mechanisms and signaling logic of several individual pathways in vertebrates and invertebrates (Clouse, 2002) (Figure 5). The ligand itself is closely related structurally to animal steroid hormones. Most multicellular organisms use steroids as signaling molecules for physiological and developmental regulation. Two different models of steroid action have been described in animal systems: the gene regulation response mediated by nuclear receptors, and the rapid non-genomic response mediated by proposed membrane-bound receptors (Wand et al., 2001). Plant genomes do not seem to encode members of the nuclear receptor superfamily (The Arabidopsis genome interactive, 2000). However, a transmembrane receptor kinase BRI1, has been implicated in brassinosteroid responses (Li and Chory, 1997; He et al., 2000). *Bri1* cannot be rescued by BR treatment, this is the evidence of its essential role in a signaling pathway. The role of BRI1 was demonstrated by binding studies with radiolabeled brassinolide and mitochondrial fraction of wild-type and mutant plants, as well as from transgenic plants expressing BRI1-green fluorescent protein (GFP) fusions (Wang et al., 2001).

A yeast two-hybrid screen for proteins putatively interacting with BRI1 identified a second leucine-rich repeat receptor kinase, termed BAK1 (BRI1 associated receptor kinase1), that shares similar structure organization with BRI1 (Nam and Li., 2002). The discovery of BAK1 and the genetic and biochemical evidence showing a role of BAK1 in BR signaling and a physical interaction with BRI is intriguing. Direct physical interaction between BRI1 and BAK1 was confirmed both in yeast cells and *Arabidopsis* plants by co-immunoprecipitation experiments with target proteins (Li et al., 2002; Nam and Li, 2002). A role of BAK1 in BR signaling was demonstrated by a number of genetic experiments. Knockout mutans of *BAK1* were identified that showed a week *bri1*-like phenotype and also decreased sensitivity to BR in root inhibition assay. Finally, overexpression of a kinase-deficient mutant form of *BAK1* in *bri* led to a severe dwarf phenotype, suggesting a dominant-negative effect, most likely by the poisoning of a possible heteromeric complex between BRI1 and BAK1 (Li et al., 2002). Nam and Li (2002) proposed a model for BR signaling in which BRI1 and BAK1 exist as inactive monomers in equilibrium with an active heterodimer. BR binding stabilizes the heterodimer and results in transposphorylation of each cytoplasmic domain by its partner, leading to activate kinases that recognize and phosphorylate currently undefined downstream components. On the other hand, Li et al. (2002) hypothesize that BRI1/BAK1 interaction is more reminiscent of the TGF-β signaling pathway. They suggest, that BR binds to BRI1, which than activates BAK1 by transphosphorylation. The activated BAK1 would then phosphorylate downstream components. Both of these models are based in part on data from kinase activity of BRI1 and BAK1 expressed in yeast and bacterial cells. Thus the genetic evidence strongly supports the biochemical experiments that show a direct *in vivo* interaction between BRI1 and BAK1 in plant membranes. However, direct demonstration of a BRI/BAK1 heterodimer as a coreceptor of BR is lacking and will be required before models of BR-dependent heterodimerization and kinase activation can be confirmed. To clarify the true nature of BRI1/BAK1 interaction it will be necessary to examine

specific phosphorylation sites and mechanisms *in planta* and their dependence on BR.

After plasma membrane perception of BRs the signaling pathway begins to resemble elements of the Wingless/wnt pathway. An unknown number of steps follow BR binding, leading to inactivation of the negative regulator BIN2. Inactivation of BIN2 allows the unphosphorylated form of BES1 and BZR1 to accumulate and translocate to the nucleus. These proteins are obviously involved into the regulation of BR-responsive genes, but the mechanism remains completely unknown. By analogy with β-catenin in the Wingless/wnt pathway, BES1 and BZR1 may complex with transcription factors, and yeast two-hybrid analysis may be effective in identifying such putative binding partners. Once BR response elements have been located in the promoters of BR regulated genes, it will also be possible to conduct yeast one-hybrid analysis to isolate transcription factors associated with BR signalling. The gap between BRI1 and BIN2 represents a major deficiency in our understanding of BR signaling. Substrates for the BRI1 (and BAK1) kinase domain need to be definitively identified and their function characterized. Isolation of receptor kinase substrates will allow a second round of screening for downstream interacting components.

The dramatic phenotype of BR-deficient and insensitive mutants is clear evidence of the critical importance of BR activity in the growth and development of plants. A greater understanding of the molecular mechanisms of BR signal transduction will deepen our knowledge of plant growth and development and will establish further similarities and contrasts between plant and animal steroid signaling pathway.

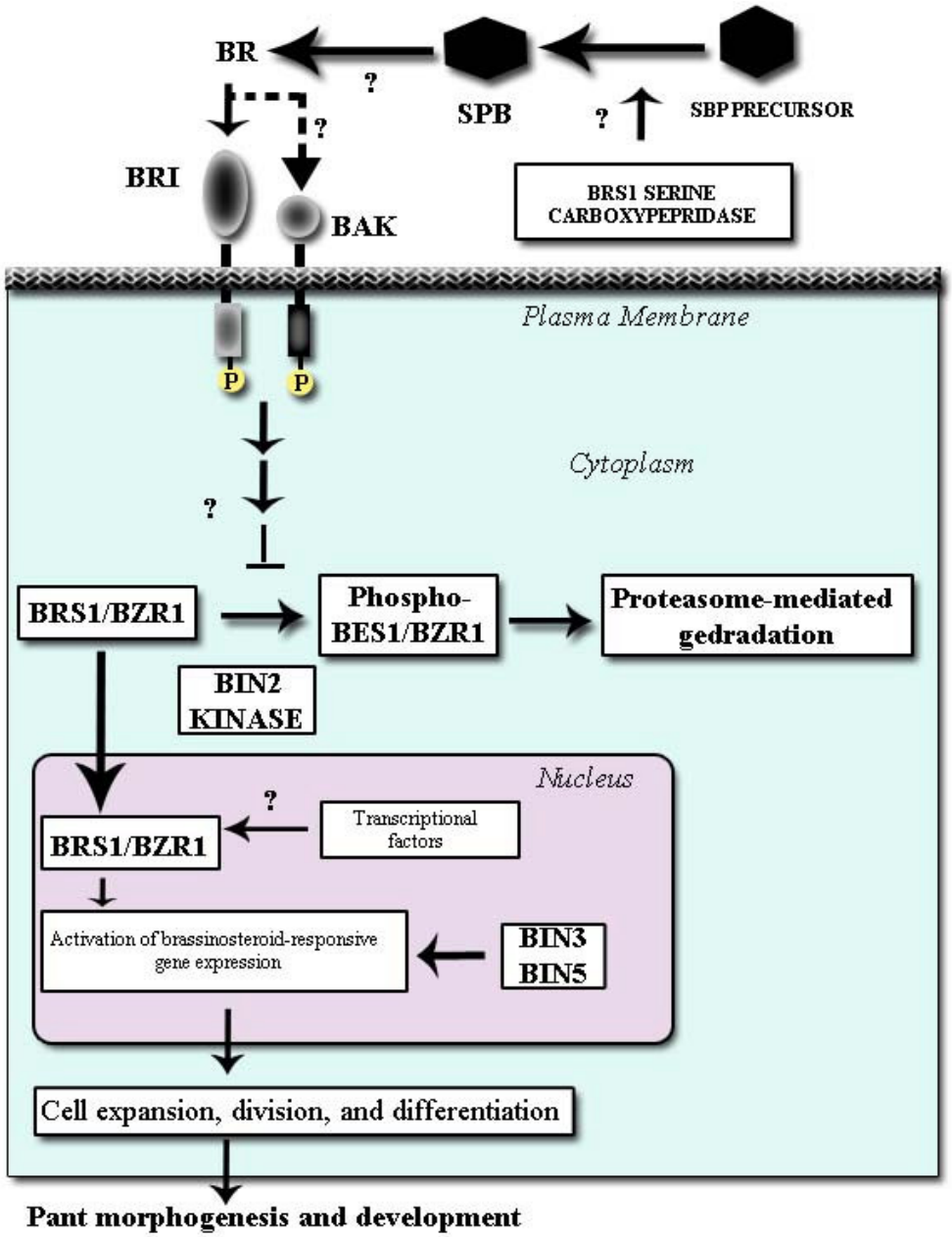

Figure 5. Model of Brassinosteroid Signal Transduction in *Arabidopsis* (Clouse 2002)

BR either binds directly to BRI1 or first complex with an unidentified steroid binding protein (SBP), which may require processing by a serine carboxypepidase. BRI1 and BAK1 have been shown to interact in vivo, but it was not yet been verified whether or not a BRI1/BAK1 heterodimer acts as a coreceptor for BR. Ligand binding initiates a signaling cascade that inactivates the BIN2 kinase and allows accumulation and nuclear localization of the unphosphorylated forms of the positive regulator BES1 and BZR1. The BIN2 kinase shares 70% amino acid identity in the catalytic domain with GSK-3/shaggy kinases of vertebrates and invertebrates. These kinases are cytoplasmic and act to negatively regulate signaling by phosphorylating positive regulators of the pathway, targeting them for proteasome-mediated degradation. Question marks represent proposed but uncharacterized steps.

1.5.2. Brassinosteroid regulated gene expression

A number of genes regulated by BR either by transcriptional or posttranscriptional mechanisms have been identified, although a BR response element and interacting protein factors have not as yet been reported (Bishop and Koncz, 2002). Plant cell expansion and division are critical for growth and differentiation in all organs and results from alterations in gene expression and biochemical processes that affect on mechanical properties of cell wall, cell hydraulics, number of cells, and the osmotic potential. Modulating the expression of genes encoding wall-modifying proteins is one mechanism by which plant hormones, such as BRs, auxins and gibberellins promote cell elongation and many of the known BRs-regulated genes encode such proteins. Another way for plant growth is the cyclin expression regulation. As it was shown by Hu et al. (2000), *CycD3* is induced by epi-brassinolide. Down-regulation of the genes encoding BR biosynthetic enzymes has been demonstrated, as has for BR-regulated genes encoding proteins associated with environmental adaptation, pathogen attack, assimilate partitioning, biosynthesis of other plant hormones, and translation initiation (Bishop and Koncz, 2002).

Most of the genes were identified by classical methods of studying differential gene expression, like subtractive hybridization. More recently, DNA microarray analysis has been employed to identify several novel genes that appear to be regulated by BR signaling. Examination of BR-regulated gene expression in the *bes1-1D* and *bri1* mutants using Affymetrix GeneChip arrays verified the BR regulation of numerous genes encoding wall-modifying proteins and showed that several genes associated with auxin signal transduction are also BR regulated (Yin et al., 2002). Interestingly, an independent genetic analysis of the *Arabidopsis ucu1* mutant (allelic to *bin2*) suggests that the BIN2/UCU1 kinase may also be involved in auxin signal transduction, indicating the possible crosstalk between BR and auxin, two hormones with pronounced effect on the cell elongation (Perez-Perez et al., 2002). A recent genetic screen for new BR-insensitive mutants, combined with Affymetrix GeneChip analysis, has identified *bin3* and *bin5* as putative subunits of an *Arabidopsis* topoisimerase VI that regulates the expression of numerous genes, including many of those that are also regulated by BRs (Yin et al., 2002). Finally, a global expression analysis using Affymetrix GeneChips and RNA from weak alleles of BR-deficient mutants grown under two conditions has identified a core set of BR-regulated genes involved in BR biosynthesis, auxin response, nitrogen transport, and transcriptional activation (Müssig et al., 2002).

While a number of BR-regulated genes have currently been identified in *Arabidopsis,* preliminary microarray experiments suggest that much more of these genes are required to drive BR-promoted developmental processes, that leads to normal plant growth (Clouse, 2002). The recent availability of full-genome *Arabidopsis* GeneChip from Affymetrix (recognizing more than 24000 distinct genes) provides a unique opportunity to characterize the full spectrum of BR-regulated genes. Like other plant hormones, the effect of BRs on growth are pleiotropic and the cataloging and functional analysis of newly discovered gene sets may increase our understanding of the range of physiological events influenced by BRs.

1.6. Immunomodulation.

Immunomodulation is a molecular technique that allows to inhibit or to modulate functions of corresponding antigens *in vivo* by intracellular ectopic expression of specific antibodies or antibody fragments. The modulation is

specifically due to interaction of antibody and antigen by formation of antigen-antibody complexes. Antibody binding can interfere with enzyme-substrate interaction, changes quaternary structure of an antigen, causes allosteric inhibition of active protein, or the antibody binds to substrate or ligand itself (De Jaeger et al., 2000). After biosynthesis, light and heavy chains of the antibody assemble to the functional antibody molecule by formation of disulphide bridges. The problem of a correct assembly of antibody chains at ectopic expression in transgenic cells can be resolved by using single chain fragment variable (scFv) antibodies. These genetically engineered antibodies, consisting of variable light and variable heavy chain domains connected by flexible short linker polypeptide, can be expressed intracellularly without specific requirements for chain assembly as necessary to form a complete immunoglobulin. Single chain variable fragments antibodies retain full antigen-binding specificity and activity (Conrad and Fiedler, 1998).

Creation of phage scFv libraries of human or animal variable immunoglobulin gene repertories using RT-PCR and phage display technology simplified the isolation of specific scFv cDNA sequences (Kramer, 1998). cDNA sequences coding scFv can be fused with corresponding target peptides for compartment-specific localization of the scFv in transgenic cells to modulate the corresponding target antigen in different cell compartments as cytosol, ER and apoplastic space (Conrad and Fiedler, 1998). With use of seed specific promoters like LeB4 and USP, scFv proteins can be synthesized later or earlier during seed development (Fiedler et al., 1997)

The technology of plant-derived antibodies has an increasing number of applications. One aspect is the utilisation of „plantibodies“ in immunotherap y for production of immunoconjugates, for production and storage of vaccines or for passive immunization with secretory IgA (Ma and Hein, 1995). However the plantibody approach can go far more beyond the use of plants as bioreactors of therapeutically interesting molecules.

A very large area has been opened with the molecular technique of immunomodulation, which is a technique that allows the interference with cellular metabolism or pathogen infectivity by the ectopic expression of genes encoding antibodies or antibody fragments. Important prerequisites of the immunomodulation approach are: first, the availability of a specific antibody with high affinity for the target molecule, and second, an accumulation of the antibodies in high concentration in the right plant organ and plant cell compartment.

The potential mechanisms of antibody-mediated *in vivo* modulation of proteins and signal molecules is reviewed in (De Jaeger et al., 2000). The application of recombinant antibodies in biological studies is almost unlimited as they can compete with substrate, hormone or secondary metabolite. They can bind to a functional protein and affect the protein structure, may mistarget low molecular weight compounds, bind the substrate, hormone or secondary metabolite, interfere with DNA binding or with multiprotein-complex formation. Several experiments have been performed according pathogen-specific antibody expression in plants. We can mention the cases when resistance to artichoke mottled crinkle virus (Tavladoraki et al., 1993), tobacco mosaic virus (Voss et al., 1995) or necrotic yellow vein virus (Fecker and Koenig, 1999) were obtained. Plantibody immunization is also an elegant strategy to control mycoplasma infection, and challenging alternative to control resistance against multicellular pathogens, such as nematode, fungi or insect (De Jaeger, 2000).

In order to study physiology and development of plants, several strategies are used to analyze the functional activities of regulatory compounds in metabolic or

signal pathways. As molecular biological methods, sense, antisense RNA approaches and RNA-mediated interference are useful tools to affect the target gene in the cells of transgenic organism to create mutants. However, these methods are not successful in all cases, require special promoter activities with respect to organ-, tissue- or cell-specificity, and are not suitable for compartment-specific functional analysis or functional investigations of protein encoded by multigene families. In contrary, scFv can be expressed developmentally regulated and with organ-, tissue-, cell or compartment-specificity. Additionally, scFv, specific to gene products encoded by a whole gene family, are able to immunomodulate functional activities of all encoded proteins, whereas scFv directed specifically against one member of the gene family will inhibit individual isoforms (Conrad and Manteuffel, 2001).

Originally developed for human cells (Marasco 1995, Cardinale et al., 1998, Cattaneo and Biocca, 1999), immunomodulation was also successfully used to study the functions of phytohormones abscisic acid and gibberellin in transgenic plants (Artsaenko et al., 1995, Shimada et al., 1999, Conrad and Manteuffel 2001) and plant regulatory receptor protein phytochrome (Owen et al., 1992).

1.7. Aim of the work

The main target of this study was to open a door to the way of understanding the functions of BRs in the seeds with a use of immunomodulation methods. For this purpose scFv antibody against BRs were expressed in the seeds of *Arabidopsis thaliana*. As it is known, the biggest concentration of BRs occur in the immature seeds (Takatsuto 1994) and almost all BRs mutant are not producing seeds. Our main interest of our study was concentrated to the seed germination and future seedling development. A delay in germination, roots and seedling development was observed on plants growing *in vitro*. Therefore, the molecular characterization of observed effects the Affymetrix analysis were done. In addition to the gene expression characterization microscopic and protein analyzes were included to observe the change which occur in the immunomodulated seeds. From this data we could select the groups of genes with changed expression. We propose a model of BRs functioning in the seeds development and which leads to visible effects during germination.

2. Materials and methods

2.1. Materials

2.1.1. Bacterial strains and Phages

Escherichia coli:

XL1- Blue MR	*recA1, endA1 gyrA96 thi-1, hsdR17, supE44 relA1 lac[F´proAB lac1^{q}ZΔM15 Tn10 (Tet^r)]*
HB2151	*K12, ara, Δ(lac-pro), thi/F' pro A$^+$B$^+$, lac1^{q}ZΔM15.*

Agrobacterium tumefaciens:

C58C1	(pGV2260 in C58C1; Deblaere *et al.* 1985)

2.1.2. Vectors

pRTRA7/3 (Artsaenko, 1996)	amp^r
pK11	amp^r
PBIN19 (Bevan et al., 1984)	km^r

2.1.3. ScFv genes in pIT vector

anti-24-brassinolide scFv gene A30 (Fecker, unpublished)
anti-24-epi-brassinolide scFv gene A16 (Fecker, unpublished)

2.1.4. Plant material

Nicotiana tabacum cv. Samsun NN,
Arabidopsis thaliana (ecotype Columbia, Col-0)

2.1.5. Oligonucleotide primers

2.1.5.1. Oligonucleotide primers for PCR amplification

12SF – 5' – ACA AGG GTT CCG TGA CAT – 3' (Metabion GmbH, D)
12SB – 5' – AGC CTG ACA TAC TCC AAG AT – 3' (Metabion GmbH, D)
HSP17F - 5' – CGT TTT CGC CGT GGA CAT – 3' (Metabion GmbH, D)
HSP17B - 5' – CTT GGG TTT CTT GGC TCA GG – 3' (Metabion GmbH, D)
EFF – 5' –GCT GTT CTT ATC ATT GAC TCC ACC ACT – 3' (Metabion GmbH, D)
EFB - 5' – GGC ACC GTT CCA ATA CCA CCA AT – 3' (Metabion GmbH, D)
BAM1 - 5' – GTC ACG GGA TCC ATT CAG ATC CTC TTC TGA GAT GAG TT – 3' (Metabion GmbH, D)
BAM2 - 5' – AGA CGT GGA TCC GAG GTG CAG CTG TTG GAG TCT GGG GGA GGC TTG GTA CAG CCT GGG G – 3' (Metabion GmbH, D)
FORTOM - 5'-GTC ACG GGA TCC ATT CAG ATC CTC TTC TGA GAT GAG TT - 3' (Amersham, D)

BACKLEGTOM - 5'-ACA CGT GGA TCC GAG GTG CAG CTG TTG GAG TCT GGG GGA GGC TTG GTA CAG CCT GGG G -3' (Amersham, D)

LeBF - 5'-GTA AAA CCA TGA CAT CCA CTT G-3' (Metabion GmbH, D)

A16LeB - 5'-TGC TAC CAC CAC TAC CAC TAA TA-3' (Metabion GmbH, D)

USPF - 5'-GAA CTA AAG ATT TGC TGC TAC ACG-3' (Metabion GmbH, D)

A16USP - 5'-CGG CCC TTC ACG GAG TC-3' (Metabion GmbH, D)

2.1.5.2. Oligonucleotide primers for DNA sequencing

35S: 5'- CAC TGA CGT AAG GGA TGA CGC -3' (http://pgrc.ipk-gatersleben.de/export/protocols/primer_abi.php)

LEG 73 - 5'-GCT TTC ACT TTC CTT GCT TC-3' (Boehringer/Mannheim)

TAG 73 - 5'-GAG AGA CTG GTG ATT TTT GCG-3' (Boehringer/Mannheim, D)

M13-21uni - 5'-GTA AAA CGA CGG CCA GT-3' (http://pgrc.ipk-gatersleben.de/export/protocols/primer_abi.php)

M13-24rev - 5'-AAC AGC TAT GAC CAT G-3' (http://pgrc.ipk-gatersleben.de/export/protocols/primer_abi.php)

LEG 73 - 5'-GCT TTC ACT TTC CTT GCT TC-3' (Boehringer/Mannheim, D)

TAG 73 - 5'-GAG AGA CTG GTG ATT TTT GCG-3' (Boehringer/Mannheim, D)

2.1.6. Media

All media are calculated for a volume of 1l and were sterilized by autoclaving for 20 min at 120 C. Antibiotics, hormones or other components, specified in chapter 2.2, were added after autoclaving the media.

2.1.6.1. Media for plant culture

MS medium (Murashige and Scoog, 1962):

4.49g Murashige and Scoog medium basal salt mixture including vitamins and microelements (Duchefa, Haarlem, The Netherlands),
30g sucrose pH 5.8,
1% agar (Difco, Detroit, USA) for solid medium.
Sterilized by autoclaving.

2.1.6.2. Media for bacterial culture

SOB medium:

Tryptone 20g,
yeast extract, 5g,
NaCl, 0.5g
Adjust the pH to 7.0 with 5N NaOH.
Sterilize by autoclaving. Just before use, add 5ml of a sterile solution of 2M $MgCl_2$. (This solution is made by dissolving 19g of $MgCl_2$ in 90ml of deionized H_2O. sterilize by autoclaving).

LB medium:

5g Yeast extract (Difco),
10g Tryptone (Difco), 10g NaCl,
10g agar (Difco) for solid medium,

pH 7.0
Sterilized by autoclaving.

SOC medium:

5g Yeast extract (Difco),
20g Tryptone (Difco),
0.5g NaCl,
0.186g KCl,
20mM Glucose,
pH 7.0
Sterilized by autoclaving.

2×TY medium:

16g Tryptone (Difco),
10g Yeast extract (Difco),
5g NaCl, pH 7.0,
10g agar for solid medium.
Sterilized by autoclaving.

YEB medium:

5g Beef extract (Difco),
1g Yeast extract (Difco),
5g Peptone (Difco),
5g Sucrose,
2mM $MgSO_4$,
pH 7.2
Sterilized by autoclaving.

2.1.7. Buffers

A-PBS:

- 100mM $Na_2HPO_4 \times 12H_2O$,
- 100mM $NaH_2PO_4 \times H_2O$,
- 0.15M NaCl,

pH 7.2

PBS:

- 0.03M $Na_2HPO_4 \times 2H_2O$,
- 0.017M $NaH_2PO_4 \times H_2O$,
- 0.1M NaCl,

pH 7.2

Carbonate buffer:

- 15mM Na_2CO_3,
- 35mM $NaHCO_3$,

pH 9.6

Denaturation solution:

- 1M NaCl,
- 0.4M NaOH.

EB buffer:

- 1M Tris-HCl,
- 1%SDS (used DEPC-treated H_2O).

pH 9.0

4×Marvel buffer:

- 80mM Tris-HCl,
- 720mM NaCl,

pH 7.8

10×MEM solution:

- 0.2M MOPS,
- 0.08M Sodium acetate,
- 5mM EDTA,

pH 7.0

SDS-sample buffer (10 ml):

- 1.4ml Stacking buffer,
- 2ml 10% SDS,
- 0.5ml β-mercaptoethanol,
- 5.1ml H_2O,
- 0.001% bromphenol blue 1ml glycerol.

Stacking buffer:

- 0.5M Tris-HCl,
- 0.4%SDS,

pH 6.8

TE-buffer:

- 10mM Tris-HCl, pH 8.0,
- 1mM EDTA.

TAE-buffer:

- 0.04 M Tris-acetate,
- 0.001M EDTA,

pH 8.0

Tris-Saline buffer:

- 0.01M Tris-HCl,
- 0.15M NaCl,

pH 7.4

Alkaline phosphatase substrate buffer:

- 100mM Tris-HCl,
- 100mM NaCl,
- 5mM $MgCl_2$,

pH 9.5

20×SSC buffer:

- 175.3g NaCl,

- 88.2g Sodium citrate,

pH 7.0

Church buffer (Church and Gilbert, 1984):

- 0.5M Sodium phosphate buffer (0.5M NaH2PO4 and 0.5M Na2HPO4) pH 7.0,
- 2mM EDTA,
- 1% BSA,
- 7% SDS

TBW1 buffer:

- 100mM KCl,
- 50mM $CaCl_2$.

Sterilized by filtering.

TRB2 buffer:

- 75mM$CaCl_2$,
- 10mM$RbCl_2$,
- 10mMHEPES,
- 15% glycerol.

Sterilized by filtering.

Extraction buffer:

- 200mM Tris-HCl pH 8.0,
- 250mM NaCl,
- 125mM EDTA,
- 0,5% SDS.

Lysis buffer:

- 10mM Tris pH 8.0,
- 10mM EDTA,
- 0,1mM NaCl.

Cacodylate buffer:

- 2% glutaraldehyde
- 3% formaldehyde,

pH 7.2

2.1.8. Enzymes

restriction enzymes Superscript ™ (GibcoBRL, D);
II Rnase H^- Reverse Transcriptase (GibcoBRL, D);
RNase A (GibcoBRL, D);
Taq polymerase (GibcoBRL, D);
T4-DNA ligase (GibcoBRL, D);
Shrimp Alkaline Phosphatase (Roche, D);
T4 DNA Ligase (Invitrogen).

2.1.9. Antibiotics

carbenicillin disodium (Cb) (Duchefa);

kanamycin monosulfate (Km) (Duchefa).

2.1.10. Immunochemicals

anti-cruciferin polyclonal serum (kindly provided by R. Manteuffel, IPK, Gatersleben, Germany);

anti-c-myc monoclonal antibody 9E10, (Tillack, Research Group Phytoantibodies, IPK, Gatersleben, Germany);

anti-mouse IgG horseradish peroxidase-linked whole molecule (IgG-HRP) (Amersham, D);

anti-rabbit IgG horseradish peroxidase-linked whole molecule (IgG-HRP) (Sigma);

24-epi-castosteron-BSA conjugate (Fecker, unpublished).

2.1.11 DNA- and proteinmarkers

DNA Smart Ladder (Eurogentech, Seraing, Belgium);
SmartLadder (Eurogentec, D);
Western blot bench marker (GibcoBRL, D).

2.1.12 Plant hormones

brassinolide [(22R,23R,24S)-2α,3α,22,23-tetrahydroxy-24-methyl-B-homo-7-oxa-5α-cholestan-6-one];

24-epibrassinolide [*24-epibrassinolide*, (22R,23R,24R)-2α,3α,22,23-tetrahydroxy-24-methyl-B-homo-7-oxa-5α-cholestane-6-one] (kindly provided by Dr. Strand, IEB, Olomouc, Czech Republic).

2.1.13 Kits

α-^{33}P-dCTP (Amersham, D);
α-^{32}P-dCTP (Amersham, D);
RediPrime Kit (Amersham, D);
ECL Western blotting analysis system (Amersham, D);
TA Cloning Kit (Invitrogen LTD);
Dynabeads® mRNA purification kit (Dynal, Norway);
Purescript RNA Isolation Kit (Gentra; USA);
RevertAid^{TM} First Strand cDNA Synthesis Kit (Fermentas; Litvania);
QIAEX II gel extraction kit (QIAGEN, D);
QIAGEN plasmid kit (QIAGEN, D);
DNeasy Plant Kit (QIAGEN, D).

2.1.14 Special laboratory reagents

Bacto-agar (Difco, USA);
Yeast extract (Difco, USA);
Bacto-peptone (Difco, USA);
DEPC (Sigma, D);
bovine serum albumin (Sigma, D);
p-nitrophenyl phosphate (p-NPP) (Sigma, D);
Bio-Rad protein assay (Bio-Rad, D);
Triton X-100 (Serva, D);
Tween20 (Serva, D);
ethidium bromide (Roth, D);
37% formaldehyde (Roth, D);
13% sodium hypochlorid (Roth, D);

formamide (Roth, D);
glycine (Roth, D);
hybridisation solution Roti-Hybri-Quick (Roth, D);
X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) (Roth, D);
Rotiphorese® Gel30 (Roth, D);
Tris (Roth, D);
Murashige-Skoog medium basal salt mixture (Duchefa, NL);
Sucrose (Duchefa, NL);
IPTG (Boehringer/Mannheim, D)
Marvel dried skimmed milk fat free (Marvel, GB)
polyethylenglycol 6000 (PEG 6000) (Merck, D)
MES AppliChem, D (GibcoBRL, D)
NBT/BCIP stable mix (GibcoBRL, D)
TEMED (GibcoBRL, D)

2.1.15 Special laboratory tools

Filter paper Whatman no.4 (Whatman®, USA);
Fuji imaging plate BAS-MS (Fuji Photo Film, Japan);
microtitre plates Falcon 3915 (Becton Dickinson and company, USA);
nylon membranes Hybond N+ (Amersham, D);
Parafilm® – laboratory film (American National Can™, USA);
ProbeQuant G-50 Micro columns (Amersham, D);
Protran® nitrocellulose transfer membrane (Schleicher & Schuell, D);
rProteinL™coupled to CNBr-activated sepharose (ACTIgen, D);
syringe filters 0.2µm Nalgene® (Schütt Labortechnik, D);
X-ray film (Retina, D).

2.1.16 Special laboratory equipment

Biofuge fresco (Heraeus, D);
ELISA reader Dynatech MR 7000, GeneAmp® PCR system 9700 (PE Applied Biosystems, USA);
Gene-Pulser™ (Bio-Rad, D);
hybridisation oven (Bachofer, D);
phosphoimager Storm860 (Molecular Dynamics, USA);
spectrophotometr Spectronic Genesys5 (Milton Roy, USA);
transfer electrophoresis unit (Schütt Labortechnik, D);
transilluminator 2020E UV/white (Stratagene, D);
ultrasonic device Sonorex RK510S (Bandelin, D);
vacuum centrifuge concentrator 5301 (Eppendorf, D).

2.1.17. Software

DNA and protein sequence data were processed using the program package *Lasergene* version 4 and 5 of DNASTAR Inc., USA and *BLAST* (Basic Local Alignment Search Tool). The autoradiography images were analyzed by TINA 2.09 of Raytest Isotopenmeßgeräte GmbH, Germany), Affymetrix Microarray Suite software version 4.0.

2.2. Methods

2.2.1. *E.coli* transformation

2.2.1.1. Preparation of competent *E.coli* cells

5ml of SOB medium was inoculated with a single colony of XL1 Blue cells and incubated overnight at 37°C. 1ml of the overnight culture was used for the inoculation of 100ml SOB medium. The culture was incubated at 37°C by shaking until the optical density at 550nm reached 0.4. Cells were centrifuged for 10 min at 4°C (4500rpm), the pellet was resuspended in 20ml ice cold solution TBW1 and let on ice for 15 min. Cells were centrifuged for 15 min at 4°C (4500rpm) and the pellet was resuspended in 2ml ice cold solution TRB2. 100µl aliquots of heat shock-competent cells were frozen in liquid nitrogen and stored at -80°C

2.2.1.2. Heat shock transformation of competent *E.coli* cells

Stored competent cells were refrozen on ice. DNA samples or aliquots of a ligation mixture (5µl), chilled on ice, were added to competent cells, mixed and incubated for 30 min on ice. The tubes were transferred to 42°C for exactly 90 sec and rapidly transferred to ice for 1-2 min. 800µl of SOC medium were added to each tube and incubated at 37°C for 45 min to allow bacteria cells to recover and to express the antibiotic marker encoded by the plasmid. Aliquots of transformed cells (up to 200µl) were transferred onto LB agar containing the selective antibiotic and grown overnight at 37°C.

2.2.1.3. Electroporation

A pulse (2.5V, 25µF, 200Ω) was applied from the Bio-Rad Gene-Pulser™ on the cuvette containing 50µl of electroporation-competent *E.coli* cells with 1µl of plasmid DNA. Cells were resuspended in 1ml of SOC medium, incubated for 45 min at 37°C and plated on TBY medium with 100mg/l Cb

2.2.2. Transformation of *Agrobacterium tumefaciens* cells

2.2.2.1. Preparation of competent *Agrobacterium tumefaciens* cells

The overnight culture of *Agrobacterium tumefaciens* in YEB medium was diluted with fresh medium 1:100 (final volume 200ml) and grown at 28°C till OD_{600} reached approximately 0.5-0.8. Cells were collected by centrifugation for 20 min at 300rpm at 4°C and resuspended gently in 10ml of sterile cold TE. The cells were spinned down for 5 min at 300rpm and resuspended in 10ml of sterile millipore water. After centrifugation for 5 min at 300rpm, cells were resuspended in 20ml of sterile cold YEB. 500µl aliquotes of the cell suspension were frozen in liquid nitrogen for further storage at –70°C.

2.2.2.2. Cold shock transformation of competent *Agrobacterium tumefaciens* cells

1-2mg of plasmid DNA was mixed with an aliquote of competent *A.tumefaciens* cells and freeze in liquid nitrogen for 5 min. Then cells were transformed to the 37°C water bath and incubated for 5 min. 1ml of LB medium were added to the cells. The cells were mixed and incubated for 2-3h at 28°C with shaking. Then 200µl of the cell suspension were spread onto selective YEB plates and incubated at 28°C for two days to receive visible colonies for further propagation.

2.2.3 Production of soluble scFv from *E.coli*

Non-suppressor strain HB2151 of *E. coli* was transformed with pIT vector containing the scFv gene. The culture, inoculated with an individual colony, was incubated overnight in 2xTY medium with 50mg/l Cb and 1% glucose. 5µl of this culture was transferred into medium 2xTY with 50mg/l Cb and 0.1% glucose and incubated in 37°C by shaking until the optical density at 600nm reached approximately 0.9. IPTG was added to a final concentration of 1mM IPTG and the culture was shaken overnight at 30°C. After spinning down the bacterial pellet, the supernatant was used in ELISA.

2.2.4 Purification of scFv from plant extract

1ml of rProteinL™coupled to CNBr-activated sepharose was transferred into a small column and washed with 20ml of A-PBS buffer. Approximately 0.1mg of seeds was extracted in SDS-sample buffer and used as a control of the scFv expression – probe A. 5-10g of the seeds were grinded in liquid nitrogen and 3 volumes of PBS containing 0.1% of Triton X100 was added. The sample was centrifuged at 4°C for 5 min (4500rpm) and the supernatant centrifuged again in 2ml-tubes at 4°C for 20 min (14000rpm). The obtained supernatant was applied on the washed protein L column. The application of the supernatant was repeated two times. 0.5ml of the crude plant extract was used as a control of the extraction – probe B. 0.5ml of the solution after purifying via the column was used as a control of the binding effectivity – probe C. The protein L column was washed with 100ml of A-PBS buffer. ScFv antibodies were eluted with 45ml of 0.1M glycine pH 2.5, into 2ml-tubes, each containing 60µl of 1M Tris. The column was washed with 100ml of A-PBS buffer followed by the application of 10ml of 22% ethanol in A-PBS buffer. A western blot analysis was performed to detect scFv in the fractions and in probes A, B and C. Fractions containing scFv antibodies were concentrated in PEG6000 for 3 hours in a dialysing tube. The protein content was determined by Bio-Rad Protein Assay.

2.2.5 ELISA with soluble anti-epibrassinolide scFv antibodies in bacterial supernatant and purified from plant extract

Wells of a 96 well microtiter plate were coated with 80µl of 10 µg/ml 24-epi-castosteron-BSA conjugate in PBS overnight and washed two times with PBS. For blocking, 90µl of 3% BSA in PBST were loaded per well and incubated for 1h at RT. Thereafter the plate was washed 3 times with PBS. 80µl of supernatant containing soluble scFv (or 80µl of plant-purified anti-brassinolide or anti-epibrassinolide scFv at concentration 1µg/ml in PBS) were loaded per well and incubated 45 min at RT. The plate was washed 2 times with PBS containing 0.05% Tween 20 to remove unbound scFv. 80µl of monoclonal anti-c-myc antibody, diluted 1:50 in 0,2% BSA in PBS, were loaded per well and incubated for 45 min followed by another two times washing with PBS containing 0,05% Tween 20. 100µl of anti-mouse IgG conjugated to ALP diluted 1:2000 in 2% BSA in PBS were loaded per well and incubated for 45 min. The plate was washed 3 times with PBS containing 0.05% Tween20. For signal generation, 100µl of 1ng ml^{-1} pNPP solution in alkaline phosphatase substrate buffer were added per well and incubated for 1 h at 37°C. Signals were measured at 405nm using an ELISA reader. The results were analyzed by Microsoft Excel 2000.

2.2.6 Western blot analysis

Leaf discs or seeds were homogenised in 80µl of SDS-sample buffer, boiled for 10 min, cooled for 10 min on ice and centrifuged at RT for 10 min (14000rpm). The protein content was determined by the Bio-Rad Protein Assay and loaded on 12.5% SDS-polyacrylamide gel next to the bench marker. The SDS-polyacrylamide gel electrophoresis and the protein transfer to Protran® nitrocellulose transfer membrane was done as described in [Sambrook et al., 1989]. For detection, the nitrocellulose filter was blocked for 2 h with 5% Marvel dried skimmed fat free milk in 1×Marvel buffer (5% MM), incubated for 2 h in anti-c-myc primary antibody diluted 1:50 in 5% MM and afterwards washed in 0.5% MM. Anti-mouse IgG-HRP diluted 1:2000 in 5% MM has been used as secondary antibody. After 1h incubation the filter was thoroughly washed twice in 0.5% MM, twice in 1×Marvel buffer and twice in PBS buffer before the visualisation with the ECL Western blotting analysis system. Expression level was estimated by comparing the signal intensity of the band of a sample on X-ray film with the signal intensity of the purified scFv used as a positive control.

2.2.7 Affymetrix analysis

2.2.7.1. RNA preparation

RNA was isolated from 6-days old seedlings, using Gentra (BioZim). RNA quality was confirmed by gel analyses and quantificated by spectrophotometry.

2.2.7.2. Affymetrix hybridisation

Affymetrix hybridization was performed in Deutsches Ressourcenzentrum für Genomforschung GmbH http://www.rzpd.de/ according to standard procedure http://www.affymetrix.com/.

2.2.7.3.Expression analysis

Expression analysis via the Affymetrix Microarray Suite software (version 4.0) was performed with standard parameters. Basic principles of Affymetrix oligonucleotide arrays were reviewed by Lipshutz et al. (1999).

2.2.8. Seed germination experiment

Seeds of wild type plants and transgenic plants were placed on Murashige-Skoog medium containing or without 50mg/l Km and either 50nM 24-epibrassinolid. Petri dishes were covered with parafilm and placed in to the dark place, temperature 4°C for 2 days. After that they were transferred to the cultivating room with conditions 16h light and 8h dark, temperature 22°C. Differences in seed germination between wild type and transgenic plants were obvious on 2^{d} day. The developmental differences from the stage of seedlings between transgenic plants and wild type were observed on 6^{th} day.

2.2.9. Plasmid preparation

Plasmid DNA extraction for sequencing was performed with the QIAGEN plasmid kit. The sequencing was done by S. König and carried out with an ALF DNA sequencer (Pharmacia, D).

2.2.10. Extraction of plant genomic DNA

2.2.10.1 The rapid plant DNA extraction, PCR grade

The rapid plant DNA extraction, PCR grade was carried out according to Edwards et al. (1991). The leaf tissue (~100mg) was grinded in liquid nitrogen and then 400µl of extraction buffer were added and the mixture was shaken for 1 min. The leaf suspension was centrifuged for 5 min at full speed and the supernatant transferred into a new tube containing 300µl of isopropanol. The DNA was collected by centrifugation for 10 min, washed twice with 70% ethanol, and resuspended in 100µl of H_2O.

2.2.10.2. Extraction of high purity plant DNA

High purity plant DNA was extracted and purified using the following protocol:

1. Grinding of 200-300mg young leaves by mortar and pestle in liquid nitrogen
2. Collecting of the powder into 15ml tube
3. Adding of 3,0ml Lysis buffer, 0,8ml 10% SDS and 20µl proteinase K
4. Incubation for 2h at 56°C and moderate shaking
5. Cooling to room temperature (RT)
6. Adding of 1ml saturated NaCl and mixing by inversion of the tube for 15sec
7. Centrifugation for 15 min at 4000rpm, RT
8. Transferring the supernatant into new tube with 10ml ethanol cooled to 20°C
9. Collecting of the precipitated DNA with sterile glass rod and transferring into 1,5ml tube containing 96% ethanol
10. Collecting the DNA by centrifugation and drying the samples
11. Resuspending in 500µl TE buffer
12. Purifying by Phenol/Chloroform extraction
13. Precipitating of the DNA by adding of 0,1 vol. 3M K-acetate, pH 5 and 1ml 96% ethanol
14. Centrifugation to collect the DNA and removing the ethanol
15. Washing twice by 70% ethanol and drying
16. Resuspending in 100-200µl Tris buffer, pH 8,0

2.2.11. Gene expression analysis

Total RNA was extracted from 2 days old *Arabidopsis* plants, using Gentra (BioZym). 2-5µg of the RNA was treated with RNAse free DNAse (Roche) and used to synthesize cDNA using the RevertAid (MBI Fermentas). First Strand cDNA Synthesis Kit (MBI Fermentas) with dT 18mer oligonucleotides. PCR was performed on the cDNA samples using standard conditions. Semi-quantitative reverse transcription-PCR analysis was achieved according to (Bauer et al., 1994). For each gene the optimal amount of cycles was determined so that expression could be analysed in the exponential phase of the PCR reaction. Primers utilised surrounded an intron if present to distinguish between cDNA and genomic amplification products. Amplification products were analysed by agarose gel electrophoresis and Southern blot hybridisation according to standard procedures.

2.2.12. Construction of expression cassettes for plant transformation

All the cloning procedures were performed according to standard techniques described by Sambrook et al. (1989). The plasmid pRTRA7/3 (Artsaenko, 1996) containing the 35SCaMV promoter, the legumin B4 signal peptide sequence, the

anti-ABA scFv gene sequence, c-myc tag, the ER retention sequence KDEL and poly-A sequences and the plant binary vector pBIN19 were used to construct the recombinant DNA for 35S anti-A16 scFv-c-myc-KDEL and 35S anti-A30 scFv-c-myc-KDEL fusion expression in plants. Plasmid pK11 containing the legumin B4 promoter, the legumin B4 signal peptide sequence, the anti-ABA scFv gene sequence, c-myc tag, the ER retention sequence KDEL and poly-A sequences and the plant binary vector pBIN19 were used to construct the recombinant DNA for LeB4 anti-A16 scFv-c-myc-KDEL and LeB4 anti-A30 scFv-c-myc-KDEL fusion expression in plants. The plasmid pRTRA7/3 (Artsaenko, 1996) containing the **u**nknown **s**eed specific **p**romoter (USP), the legumin B4 signal peptide sequence, the anti-ABA scFv gene sequence, c-myc tag, the ER retention sequence KDEL and poly-A sequences and the plant binary vector pBIN19 were used to construct the recombinant DNA for USP anti-A16 scFv-c-myc-KDEL and USP anti-A30 scFv-c-myc-KDEL fusion expression in plants. Expression cassettes were constructed by replacing the anti-ABA scFv gene sequence in pRTRA7/3 by anti-A16 scFv or anti-A30 scFv gene sequences.

For obtaining the cytoplasmic expression cassette under control of 35S CaMV promoter of anti-A16 scFv or anti-A30 scFv genes were cut from phagemids pITA16 and pITA30 using restriction enzymes NcoI and NotI. DNA fragments were electrophoretically separated on agarose gel. The 800bp NcoI – NotI fragment containing the scFv coding sequence was extracted from agarose gel using QIAGEN Gel Extraction Kit (QIAGEN) according to manufacturer's instructions and cloned into the NcoI and NotI sites of plasmid pRTRA7/3 containing 35SCaMV promoter.

In order to create the ER expression cassette under control of legumin B4 promoter and USP promoter, the anti-A16 scFv or anti-A30 scFv sequence were PCR-amplified using BAM1 and BAM2 primers containing BamHI sites at their 5'-ends. Reaction was done in 50 µl of amplification mixture containing 0.2µM primers BAM1 and BAM2, 0.2µM dNTPs, 1U Pfu Turbo DNA polymerase, 5µl 10×Pfu Buffer and 50ng of plasmid pIT1 DNA as a template with following program: 1 min 95°C, 1 min 66°C, 1 min 72°C. The PCR product was digested by use of restriction enzyme BamHI and cloned into BamHI sites of plasmid pRTRA7/3. Prior to ligation dephosphorylation of 3′-ends of pRTRA7/3 DNA was performed by adding 1U of shrimp alkaline phosphatase during digestion of plasmid DNA with restriction enzyme BamHI.

All constructed plasmids containing the scFv expression cassettes were digested with restriction enzyme HindIII. Subsequent to restriction DNA fragments were separated on the agarose gel. 1kb fragment containing the expression cassette and the anti-Bl scFv gene sequence was eluted from the agarose gel as described above and cloned into the Hind III site of the plant binary vector pBIN19. All DNA ligation reactions were performed using Rapid DNA Ligation Kit (Roche) according to the instructions of the manufacturer.

Five µl of ligation mixture were used to transform competent cells of *E.coli* strain XL1-Blue. Single colonies of transformed bacterial cells were propagated in LB medium supplemented with 100µg ml^{-1} of carbenicillin. Plasmid DNA was purified from 4ml of a bacterial overnight culture using QIAGEN plasmid kit (QIAGEN, D). Purified DNA of the plant binary vector was used to transform *A.tumefaciens* strain C58C1. After each step of the procedure the recombinant DNA was analyzed by sequencing using biotin-labeled primers 35S, LEB, TAQ 73, LB, LF, SCFV, SCFB. to proof the presence of scFv sequence and to check the reading frame.

2.2.13 Colony PCR of transgenic *Agrobacterium tumefaciens* cells

Single colonies of transformed *Agrobacterium tumefaciens* cells were plated by tooth picks on agarose plates with YEB medium containing 50µg/ml kanamycin and 100µg/ml carbenicillin and incubated for 2 days at 28°C. The bacterial cells were analyzed by PCR amplification with BACKLEGTOM and FORTOM primers. PCR results were analyzed by gel electrophoresis.

2.2.14. Transformation of *Nicotiana tabacum*

2.2.14.1. Leaf disk method

Agrobacterum tumefaciens C58C1 strain transformed with pBIN19 binary vector was grown in CPY medium containing 50mg/l Km and 100mg/l Cb. The presence of scFv gene in pBIN19 binary vector was confirmed by PCR analysis prior the transformation. Leaf discs of wild type *Nicotiana tabacum* plant, grown from seeds in sterile conditions, were incubated for 1 hour with the *Agrobacterium* culture and then incubated on Murashige-Skoog medium for another 2 days at 25°C. Infected leaf segments were transferred on Murashige-Skoog medium containing 100mg/l Km, 0.2mg/l NAA and 1mg/l BA. Leaf discs were replaced every 14 days on new medium containing these phytohormones and antibiotics until callus and shoots began to form (4-6 weeks). Developed shoots were transferred on Murashige-Skoog medium with 100mg/l Km. Plantlets with regenerated roots were used for Western blot analysis to determination scFv expression level and transferred to soil.

2.2.14.2. Propagation of transgenic plants

From plants grown in the greenhouse seeds were collected and sterilized by treatment with 10% NaOCl solution. After washing 3 times 10 min with sterile water the seeds were planted on 0.5XMS medium containing 50µg ml^{-1} kanamycin. Drug resistance was recognized by the ability of plants to survive the presence of the antibiotic in the medium. Resistant plantlets were propagated to establish transgenic plant lines.

2.2.15. Plant transformation of *Arabidopsis thaliana* by floral dip

Transformation of *Arabidopsis* was performed based on the protocol of Clough S.J. et al., (1998). Plants of *Arabidopsis thaliana* were grown for three weeks under short day conditions (8 hours light, 16 hours dark) and transferred to long day (16 hours light, 8 hours dark). After three weeks, the emerging bolts were cut to induce growth of multiple secondary bolts. Floral dip of plants with *A. tumefaciens* culture was done one week after clipping. Bacteria were grown until $OD_{600} > 2.0$, harvested by centrifugation and resuspended in three volumes of infiltration medium (OD_{600} approx. 0,8). Entire shoots of the plants were submerged into the *A.tumefaciens* suspension in a beaker for 5 seconds. Plants were removed from the beaker, placed on their side and kept at high humidity under plastic warp for 24 hours, after that they were uncovered and set upright. Seeds were harvested from the siliques, sterilized by Na-hypochlorite as described before and plated onto GM selection plates containing 50mg/l kanamycin. After two weeks, kanamycin resistant plants were transferred to soil, grown up and their seeds were collected. The insertion of transgene was controlled by PCR.

2.2.16. Characterization of transgenic plants

2.2.16.1. Characterization of transgenic plants by Western blot analysis

For Western blot analysis, leaves or seeds of plants were extracted with 50mM Tris-HCl buffer pH 7.6 supplemented with 150mM NaCl, 5mM EDTA, 0.1% SDS, and 0.1% ß-mercaptoethanol 1mM. Protein extracts were centrifuged at 10000***g*** for 10. The protein concentration of the supernatant was measured according to Bradford (1976). Western blot analyses were carried out as described in 2.2.6.

2.2.16.2. Quantitative detection of scFv expression by Western blot analysis

Preparative amounts of the soluble scFv were prepared by way of IPTG-induced expression in 5ml of the *E.coli* bacterial cell culture as described in 2.2.3. The supernatant of bacterial culture containing soluble scFvs was loaded on a Protein L sepharose (Amersham Pharmacia Biotech) column. After 1h incubation the column was washed with PBS buffer to remove unbound proteins. ScFv antibodies bound to Protein L Sepharose were eluted with glycine buffer at pH 2.2. The eluate containing scFvs was immediately neutralized with Tris-Saline buffer and concentrated by using collodium bags and PEG 20000. The protein concentration of the scFv preparation was measured according to Bradford (Bradford, 1976). Protein extraction from the leaf samples of transgenic plants was performed as described in 2.2.16.1. The extraction fractions were combined and used for determination of total protein concentration according to Bradford (Bradford, 1976). The pellet fraction of the samples were extracted with Laemmli buffer to check the efficiency of the extraction procedure as described in 2.2.16.1. Extracts from transgenic plants with known protein content and scFv preparations in a concentration range of 5µg, 10µg and 20µg were simultaneously separated on 12.5% polyacrylamide gel followed by Western blot analysis as described in 2.2.6. using anti-c-myc antibody as primary antibody and the ECL Kit for signal generation. The Hyperfilm image of the Western blot membrane was scanned to create a digital picture for further computer analysis. The signal intensity, generated by known amounts of scFv standard on the membrane, was determined by Tina v2.08 software (Raytest, Sprockhow, Germany) and used to calculate the calibration curve. The signal intensity generated by protein extracts from transgenic plants was also measured by Tina v2.08 software and used to calculate the content of scFv in the plant protein extracts.

2.2.17. Electron microscopy

Prior to fixation the seed coat was locally perforated with a sharp needle to facilitate access of the fixation and embedding solutions. Seeds were subsequently transferred into 0.05M cacodylate buffer. After degassing the samples were stored in a fridge overnight. On day two the seeds were washed three times 15 min with 0.05M cacdylate buffer, followed by dehydration in an ethanol series of 30%, 50%, 70%, 90% ethanol, each step lasting 15 min, and two times 30 min with 100% ethanol. After two more changes with propyleneoxide the seeds were incubated with 30% Spurr resin in propyleneoxid and left overnight at RT. The next day the seeds were 6 h infiltrated with 65% Spurr in propyleneoxid followed by overnight infiltration with 100% Spurr. On day 4 the seeds were transferred to pointed beamcapsules filled with Spurr and polymerized in an oven at 60°C. Ultrathin (95nm) sections were collected on 75 mesh hexagonal grids. After poststaining with 4% aqueous uranylacetate grids were examined in a Zeiss 902 electron microscope and digital images taken with a CCD camera.

3. Results

3.1. Construction of scFv-expression cassetes for plant transformation

Over-accumulation of hormone-specific antibodies in particular plant organs is a powerful tool for controlling a certain developmental stage of a plant. Alterations in different stages of seed maturation has been reported expressing antibody constructs under the control of the USP promoter (Phillips et al., 1997) or the legumin B4 promoter (Fiedler et al., 1997). Depending on the promoter region of the expression cassette, antibodies can be potentially expressed in any plant organ. Protein trafficking in eukaryotic cells is guided by signal sequences. At the cellular level, the effect of the antibodies is directed using such recognition sites in the expression construct. Proteins without a signal peptide on their amino-terminal end will localize in the cytosol.

For immunomodulation of brassinosteroids functions in seeds two scFv antibodies were used: A30 scFv antibodies, which bind brassinolide, castasteron and there isomer forms 24-epicastasteron and 24-epibrassinolide, and A16 scFv antibody which binds 24-epicastasteron and 24-epibrassinolide (Figure 1). They are collectively named anti-Bl scFv antibodies and were selected and characterized by L. Fecker (unpublished data) from screening Tomlinson's Human synthetic VH+VL scFv phagemid libraries A and B (for detailed protocol see web site http://www.mrc-cpe.cam.ac.uk). This library is one of the libraries of cloned human or animal variable immunoglobulin repertoires which have been constructed and used for the selection of scFv specifically binding to various antigens. The Tomlinson libraries contain a huge collection of 10^9 different phagemids each coding for a unique scFv.

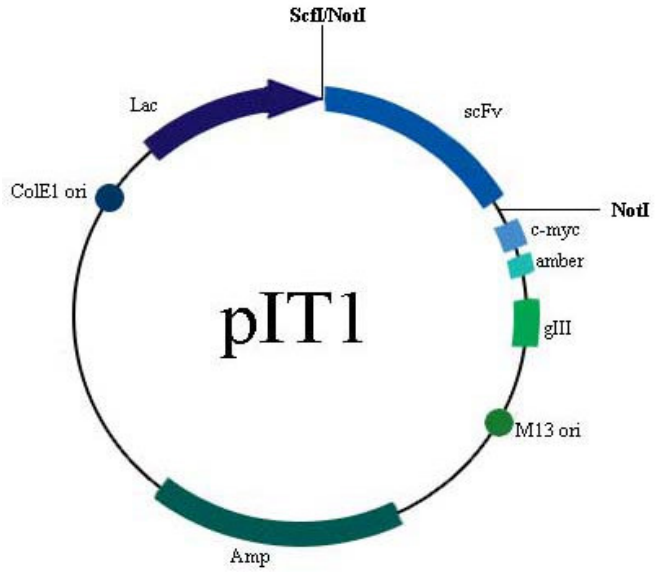

Figure 6. Schematic presentation of phagemid vector pIT1 from scFv phage library.

ScFv – scFv cDNA sequence, **Lac** – Lac Z promoter of E.coli, **pIII** – sequence of M13 phage protein pIII, **c-myc** – c-myc-tag, **amber** – amber stop-codon, **ColE ori** – ColE1 origin of replication, **M13 ori** – phage M13 origin of replication, **Amp** – ampicillin resistance gene. Restriction sites NcoI and NotI used for re-cloning of anti-ABA scFv cDNA sequence into the expression cassette pRTRA7/3.

The pIT1 vector, illustrated in Figure 6, contains an inducible lacZ promoter. The scFv gene is inserted between SfiI – NotI restriction sites. The c-myc-tag sequence is recognized by the 9E10 antibody and used for the detection of secreted scFv antibodies. Between tag-sequence and a gene coding for gIII minor phage coat protein an amber codon was introduced. The scFv can be produced either for display on phage particle from infected amber suppressor strain or secreted into the periplasm as a soluble fragment from infected non-suppressor strain.

In order to immunomodulate functions of brassinosteroids in seeds, several recombinant DNA constructs were designed with respect to overexpress anti-Bl scFv in the transgenic plants. Two recombinant DNA constructs under control of 35S CaMV promoter were created for cytosolic overexpression of anti-Bl scFv four constructions for seed specific endoplasmic reticulum (ER) overexpression of anti-Bl: two under USP promoter control and two under legumin B4 promoter control. Phagemid vectors pIT1 containing anti-Bl scFv (Figure 6); plasmids pRTBU, pRTRA7/3 (Artsaenko, 1996) (Figure 7); and pK11 (Figure 8) were used as a basis for construction of expression cassettes responsible for biosynthesis of the scFv-KDEL fusion protein in the plants.

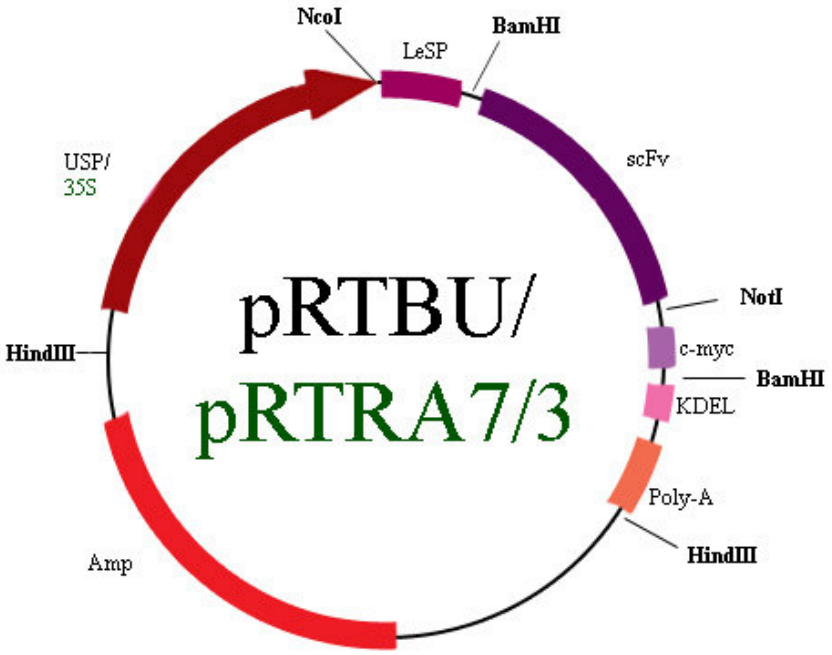

Figure 7. Schematic presentation of plasmid vector pRTBU and pRTRA7/3.

ScFv – scFv sequence, **35S** – CaMV 35S promoter; **USP** – USP promoter, **c-myc** – c-myc tag, **LeSP** – legumin B4 signal peptide sequence, **KDEL** – KDEL retention sequence, **poly-A** – polyadenine sequence, **Amp** – ampicillin resistance gene. Restriction sites NcoI, NotI and BamHI used for cloning of anti-Bl scFv cDNA into the expression cassette.

For the expression of anti-Bl in the ER under control of USP promoter vector pRTBU, containing anti-ABA scFv gene-c-myc fusion under control of the USP promoter, with the legumin B4 signal sequence and the KDEL retention sequence has been used. The vector was cleaved with BamHI, dephosphorylated and 3200bp

fragment was ligated with anti-Bl scFv genes. The BamHI restriction sites were incorporated into the scFv gene inserts by PCR with FORTOM and BACKLEGTOM primers. The PCR product, 800bp long, was cleaved with BamHI prior the ligation. XL1 Blue strain of *E.coli* was heat shock-transformed with the ligation product and ampicilin-resistant transformants were tested for the presence of scFv insert in the right orientation by NcoI-NotI cleavage. The clones with a correct orientation were sequenced with M13-21uni and M13-24rev primers and also with LEG73 and TAG73 primers. The results of sequencing confirmed correct orientation and correct reading frame of the cloned scFv genes. Overview of the cloning procedure and the final expression cassette for retention of anti-Bl scFv antibody fragments in the ER of plant cells under USP promoter is illustrated at Figure 9a.

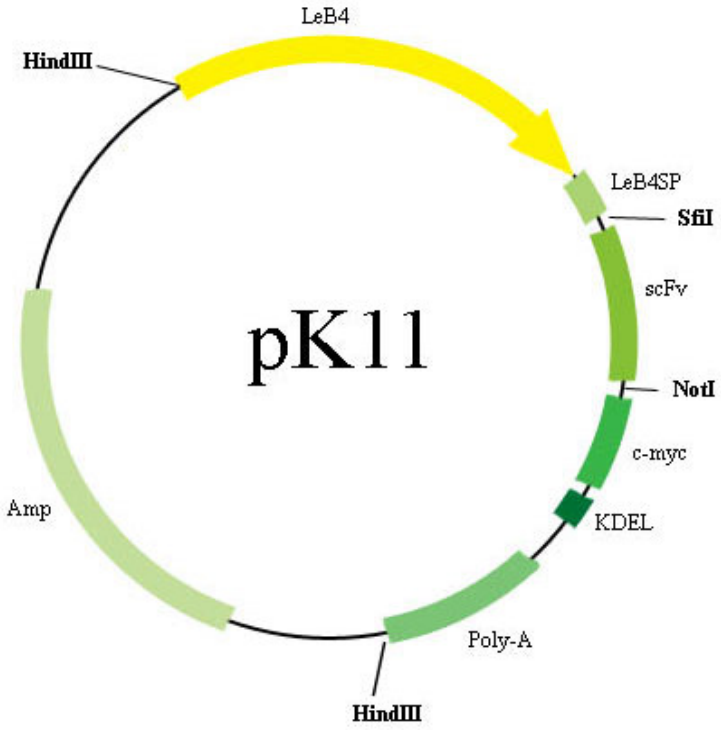

Figure 8. Schematic presentation of plasmid vector pK11.

ScFv – scFv sequence, **LeB4** – legumin B4 promoter, **c-myc** – c-myc tag, **KDEL** – KDEL retention sequence, **LeB4SP** – legumin B4 signal peptide sequence **poly-A** – polyadenine sequence, **Amp** – ampicillin resistance gene. Restriction sites Sfi I, and NotI used for cloning of anti-Bl and anti-epiBL scFvs cDNA into the expression cassette.

For the expression anti-Bl for ER retention under control of LeB4 promoter, vector pK11, containing anti-ABA scFv gene-c-myc fusion under control of the LeB4 promoter, with the legumin B4 signal sequence and the KDEL retention sequence has been used. The vector was cleaved with SfiI and NotI, dephosphorylated and 3200bp fragment was ligated with anti-Bl scFv genes. The XL1-Blue strain of *E.coli* was heat shock-transformed with the ligation product and ampicilin-resistant transformants were tested for the presence of scFv insert. The positive clones were sequenced with M13-21uni and with LEG73 and TAG73 primers. Figure 9b illustrates the schematic overview of the cloning procedure and the final expression cas-

sette for retention of anti-Bl scFv antibody fragments in the ER of plant cells under control of LeB4 promoter.

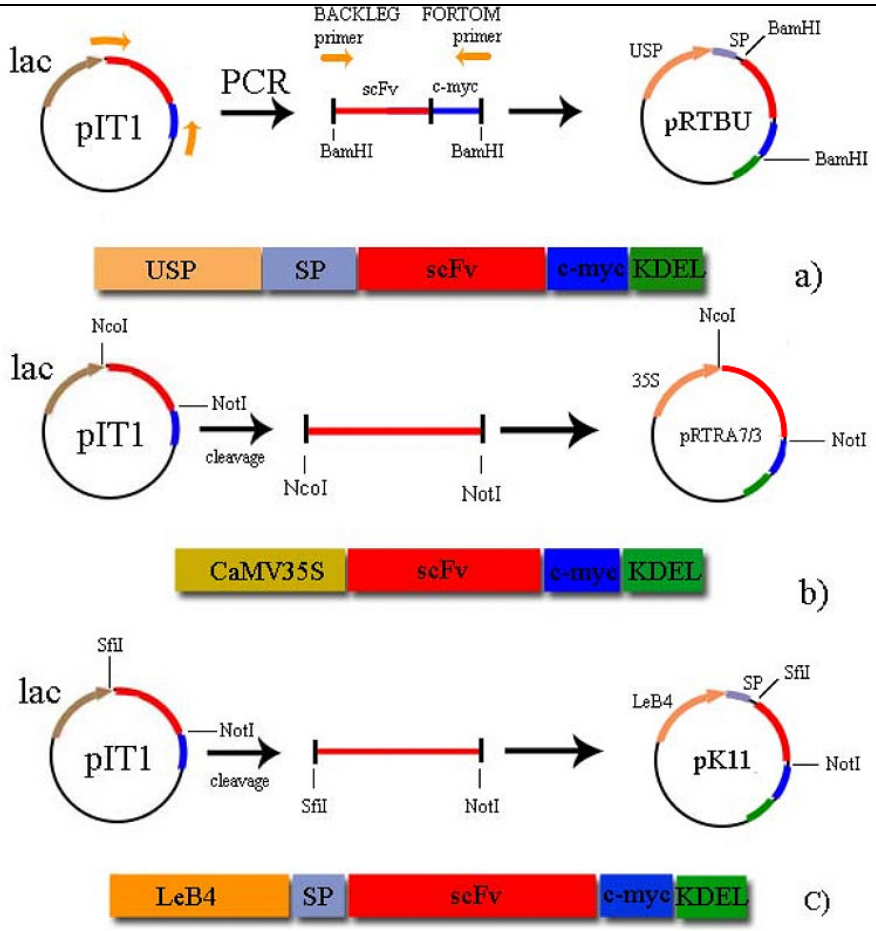

Figure 9. Schematic overview of anti-Bl scFv genes cloning and the final expression cassettes.

The expression of the scFv antibody fragments: a) in the ER of seed cells under control of USP promoter; b) in the cytosol of plant cells under control of CaMV35S promoter; c) into the ER of seed cells under control of LeB4 promoter.

For over-expression of two anti-Bl scFv genes in the cytosol, the coding sequences were cloned behind the CaMV 35S promoter into the pRTRA7/3 expression cassette lacking the LeB4 signal peptide. The most difficult is to obtain high antibody accumulation in the cytosol because of the chaperones lack and the reducing environment, which prevents the formation of disulfide bridges (Biocca et al., 1995). The KDEL peptide has been suggested to protect sterically the scFv region susceptible to proteolysis. KDEL is C-terminal (Lys-Asp-Glu-Leu) sequence that causes retention of proteins to the ER. The KDEL receptor is located mainly in the *cis*-Golgi network and in ER-to-Golgi transport vesicles; its chief function is to bind proteins with the KDEL recognition sequence and return them to the ER (Semenza et al., 1990). The tetrapeptide was fused downstream the c-myc in the construct. The anti-Bl scFv genes were cleaved from pIT1 phagemid by double restriction NcoI-NotI and directly ligated behind the promoter region of the pRTRA7/3 vector. Figure 9c

illustrates the schematic overview of the cloning procedure and the final expression cassette for retention of anti-Bl scFv antibody fragments in the cytosol of plant cells.

For *Agrobacterium*-mediated gene transfer the pBin19 expression cassettes were recloned from *E.coli* vectors to the binary vector which can be amplified in *A.tumefaciens* cells and in *E.coli* cells. Cassettes for cytoplasmic scFv expression and for accumulation of scFv in the ER were recloned to HindIII site of plasmid pBIN19. The correct reading frame of the scFv-KDEL-c-myc fusion sequence in pBIN19 was confirmed by sequence analysis using 35S and M13-21uni primers before *Agrobacterium*-mediated plant transformation was performed.

3.2. Production of transgenic plants

Plant transformation with the recombinant pBIN19-scFv constructs was performed in two steps: transformation of *A.tumefaciens* and then infection of *N.tabacum* leaf disks or floral dip of *Arabidopsis thaliana* with transgenic *A.tumefaciens* cultures.

Transformation of *A.tumefaciens* strain C58C1 was performed using the cold shock method. Several single colonies of transgenic *A.tumefaciens* grown on selective medium were transferred to fresh medium to obtain bacterial culture for plasmid DNA isolation and proof the presence of scFv DNA sequences in transformed bacterial cells by HindIII restriction cleavage and PCR amplification. The DNA from non-transformed *A.tumefaciens* cultures was used as a negative control. Transformed *Agrobacterium* clones containing the anti-Bl scFv sequences were used to infect leaf disks of *N.tabacum* plants or to make a floral dip of *Arabidopsis thailana* plants.

The leaf disks of *N. tabacum* plants were infected with a freshly grown bacterial culture of transgenic *A. tumefaciens* and after 2 days transferred to the selective medium containing antibiotic and plant hormones NAA and BA. During incubation of the leaf disks on the medium with plant hormones, callus formation was observed. The regeneration of plants from the callus started after 3 weeks of *in vitro* cultivation. Regenerated plants were cut from the callus and grown on the selective medium without plant hormones. Rooted plants were able to survive on the selective medium and can potentially be counted as transgenic plants. Fifty kanamycin resistant plants from each transformation experiment using 6 different constructs were planted in the greenhouse and propagated for flowering and seed development.

The *Agrobacterium* floral dip method allows to transform *A. thaliana* without plant tissue culture or regeneration. The optimal growth stage for transformation was when plants had numerous unopened floral buds. In this growth stage, plants were dipped into infiltration medium containing *Agrobacterium*. 20 kanamycin resistant plants were selected *in vitro* and than planted in the greenhouse and propagated.

According to the intracellular distribution of expressed scFv and promoter regulation the groups of transgenic plants were designated as followed:

<table>
<tr><td>RTA16</td><td rowspan="2">CaMV 35S promoter</td><td rowspan="2">-</td><td>A16</td><td rowspan="8">c-myc – KDEL fusion protein</td><td rowspan="2">cytoplasmic expression</td></tr>
<tr><td>RTA30</td><td>A30</td></tr>
<tr><td>TBU16</td><td rowspan="2">USP promoter</td><td rowspan="4">Signal peptide</td><td>A16</td><td rowspan="4">ER retention</td></tr>
<tr><td>TBU30</td><td>A30</td></tr>
<tr><td>TAK16</td><td rowspan="2">LeB4 promoter</td><td>A16</td></tr>
<tr><td>TAK30</td><td>A30</td></tr>
</table>

Seeds from the F_1 plants were collected and used for further propagation of the next plant generations after Km selection of germinating seeds on selective MS

medium containing kanamycin followed by propagation of Km-resistant plants in the greenhouse.

3.3. Western blot analysis of scFv expression in transgenic plants

Transgenic plants, expressing anti-Bl antibodies, were screened for transformants with high levels of scFv accumulation in different plant cell compartments. The selection was performed by western blot analysis.

In order to select transgenic plants with high levels of anti-Bl scFv expression in the cytosol, seed material from each transgenic plant was semiquantitatively analyzed by Western blotting using the anti-c-myc monoclonal antibody 9E10 as primary antibody followed by anti-mouse IgG-HRP conjugate. The signals were generated by chemiluminescence using ECL The results of Western blot analysis of protein extracts isolated from plants with a high level of anti-Bl scFv expression are shown in Figure10a for *A.thaliana* plants, in Figure 10b for tobacco.

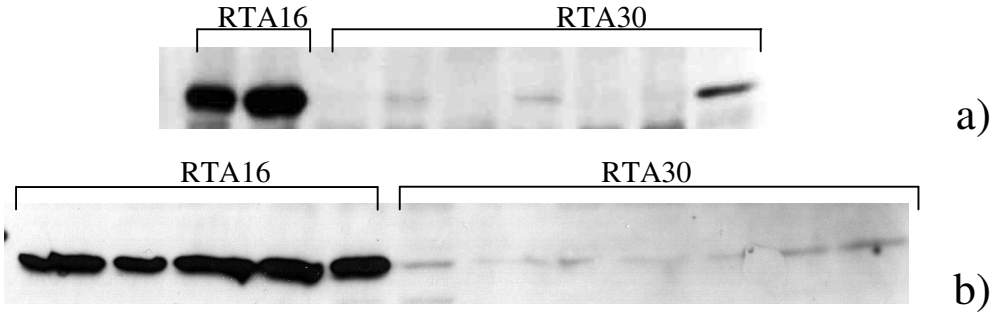

Figure 10. Detection of scFv in protein extracts of the seeds from transgenic plants of the F_1 generation by Western blot analysis

a) Seed extracts from *A. thaliana* plants; b) seed extracts from tobacco plants. **RTA16** – CaMV 35S promoter - cytoplasmic expression of anti-Bl scFv A16 – c-myc – KDEL fusion protein; **RTA30** – CaMV 35S promoter - cytoplasmic expression of anti-Bl scFv A30 – c-myc – KDEL fusion protein.

The level of cytoplasmic scFv expression varied significantly in the leaves of individual F_1 transformants, but highest scFv amounts were found in leaf extracts from plants with a construct containing anti-Bl A16 scFv gene (Table 2, 3). In comparison to RTA16 transgenics, the RTA30 plants had lower expression level. The accumulation of scFv A16 in cytosol reached more than 0,5% of total soluble protein (TSP) in contrast to scFv A30 accumulation – much less than 0,5% of TSP. The expression level strongly depends on individual scFv sequence. Consequently, RTA16 plants were selected for further experiments.

The level of scFv accumulation in ER of the seeds of transgenic plants with construct containing anti-Bl A16 scFv gene was detected in higher amount than in seeds of transgenic plants containing anti-Bl A30 gene. In the LeB4 and USP plants the scFv A16 protein was expressed approximately on the level over 1% of TSP in contrast to scFv A30 accumulation mainly below 0,5% of TSP. The difference between expression level of anti-Bl scFv A16 and anti-Bl scFv A30 is seen in both TBU and TAK plants. The highest expression levels in ripe *Arabidopsis* and tobacco seeds were obtained by using LeB4 promoter. The differences in accumulation obtained due to different antibody localization and their sequences were much more significant then the difference between scFv production under control of different

promoters. Consequently, TBU16 and TAK 16 plants were selected for further experiments.

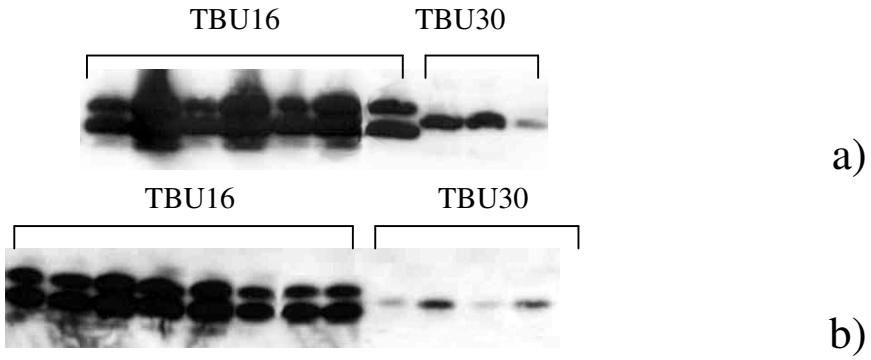

Figure 11. Detection of scFv in protein extracts of the seeds from transgenic plants of the F_1 generation by Western blot analysis

a) Seed extract from *A. thaliana* plant; b) seed extract from tobacco plants. **TBU16** – USP promoter – ER expression of anti-Bl scFv A16 – c-myc – KDEL fusion protein; **TBU30** – USP promoter - ER expression of anti-Bl scFv A30 – c-myc – KDEL fusion protein

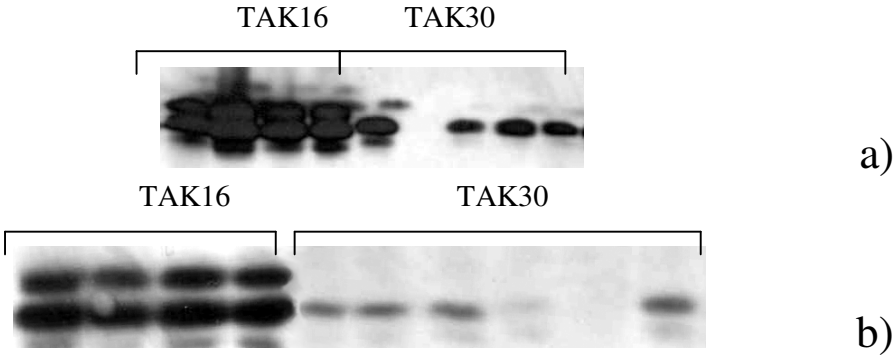

Figure 12. Detection of scFv in protein extracts of the seeds from transgenic plants of the F_1 generation by Western blot analysis.

a) Seed extract from *A. thaliana* plant; b) seed extract from tobacco plants. **TAK16** – LeB4 promoter – ER expression of anti-Bl scFv A16 – c-myc – KDEL fusion protein; **TAK30** – LeB4 promoter - ER expression of anti-Bl scFv A30 – c-myc – KDEL fusion protein

As expected, the accumulation of the anti-Bl scFv in the cytosol was significantly lower than in the ER due to the reducing environment of the cytosol as it was already described (Biocca et al., 1995). Moreover, differences in the expression level between different scFvs are obvious. The dependence of antibody intracellular stability on primary antibody sequence is known but it is not an in advance predictable phenomenon. For this reason more than one anti-Bl antibodies have been used for cloning into expression cassettes and for transgenic plants regeneration.

Table 2. Distribution of anti-Bl antibodies accumulation in the seeds between transgenic lines F_1 generation in tobacco plants.

Western blots were quantified using a pdi white light scan and the TINA-raytest program. **TBU16** – USP promoter – ER expression of anti-Bl scFv A16 – c-myc – KDEL fusion protein; **TBU30** – USP promoter - ER expression of anti-Bl scFv A30 – c-myc – KDEL fusion protein **TAK16** – LeB4 promoter – ER expression of anti-Bl scFv A16 – c-myc – KDEL fusion protein; **TAK30** – LeB4 promoter - ER expression of anti-Bl scFv A30 – c-myc – KDEL fusion protein. Expression level - **%** of total soluble protein.

Plant	**Number of the plants with expression level >1%**	**Number of the plants with expression level >0.5%**	**Number of the plants with expression level <0.5%**
RTA16	0	15	35
RTA30	0	0	50
TBU16	15	25	5
TBU30	0	5	45
TAK16	30	18	2
TAK30	0	2	48

Table 3. Distribution of anti-Bl antibodies accumulation in the seeds between transgenic lines F_1 generation in *Arabidopsis* plants.

Western blots were quantified using a pdi white light scan and the TINA-raytest program. **TBU16** – USP promoter – ER expression of anti-Bl scFv A16 – c-myc – KDEL fusion protein; **TBU30** – USP promoter - ER expression of anti-Bl scFv A30 – c-myc – KDEL fusion protein **TAK16** – LeB4 promoter – ER expression of anti-Bl scFv A16 – c-myc – KDEL fusion protein; **TAK30** – LeB4 promoter - ER expression of anti-Bl scFv A30 – c-myc – KDEL fusion protein. Expression level - **%** of total soluble protein.

Plant	**Number of the plants with expression level > 1%**	**Number of the plants with expression level > 0.5%**	**Number of the plants with expression level < 0.5%**
RTA16	0	21	4
RTA30	0	0	25
TBU16	20	0	5
TBU30	0	0	25
TAK16	23	0	2
TAK30	0	5	20

Transgenic plants with the highest expression level of scFv in the particular plant cell compartment have been chosen for further investigation and homozygous lines isolation.

3.4 Isolation of homozygous lines

In order to obtain non-segregating transgenic plant lines the classical Mendel selection was used. The recombinant plants were pre-selected by germination on MS medium containing 50 μg ml^{-1} kanamycin. The number of antibiotic resistant plants were counted and compared to the total plants number used for each transgenic plant. Seeds collected from F_1 of transgenic plants exhibited segregation of kanamycin-

resistant and kanamycin-sensitive plants with different ratios. Only lines segregating 3:1 were selected for further studies. This ratio indicated, that the active copy of the scFv gene was integrated at a single locus in the plant genome, and was inherited as a simple Mendelian trait. χ^2 calculation was done to confirm the significance of the segregation. Table 4 present an example of segregation analysis on *Arabidopsis* plants.. The selected kanamycin-resistant F_2 plants were transferred to soil and grown in the greenhouse to obtain the next generations of transgenic plants with stable scFv expression. Plants with the highest level of scFv expression were selected by western blot. The F_2 was analysed the same way and non-segregating plants were selected and propagated. The procedure was repeated with F3 as well, and here, the obtained transgenic plants were considered to be homozygous. The selected plants were analysed by western blotting for the expression level of the anti-Bl scFv in the seeds (Figure 13).

Table 4. An example of segregation analysis of F_1 on transgenic lines of *Arabidopsis* plants.

Seeds from F_1 and F_2 generations of transgenic *Arabidopsis* plants segregated on kanamycin resistant (**Km^r**) and kanamycin sensitive (**Km^s**). Selection was performed on MS medium containing 50 µg ml^{-1} of kanamycin. The seeds were germinated and cultivated for 2 weeks. **χ^2 (3:1)** – significance of 3 to 1 segregation. No * over number indicates high significance; * -lower significance; ** - very low significance.

Plant	Number of Km^r plants	Number of Km^s plants	χ^2 (3:1)	Generation
RTA16/3	94	42	2,51*	F_1
RTA163/1	62	30	2,84*	F_2
RTA163/2	78	26	0,00	F_2
RTA163/3	100	0	-	F_2
RTA163/4	100	0	-	F_2
RTA16/9	80	11	8,09**	F_1
RTA30/4	80	17	4,75*	F_1
RTA304/1	80	15	4,30*	F_2
RTA304/6	54	17	0,04	F_2
RTA304/7	64	27	1,06	F_2
RTA304/12	100	0	-	F_2
TBU16/4	36	11	0,06	F_1
TBU164/1	73	22	0,17	F_2
TBU164/8	80	25	0,08	F_2
TBU164/10	75	12	3,33	F_2
TBU164/11	100	0	-	F_2
TBU16/10	26	17	4,84*	F_1
TBU1610/2	70	13	3,86*	F_2
TBU1610/4	60	20	0,00	F_2
TBU1610/7	62	24	0,39	F_2
TBU1610/10	100	0	0	F_2
TAK16/7	63	25	0,55	F_1
TAK167/3	60	15	1,00	F_2
TAK167/6	100	0	-	F_2
TAK167/7	94	3	24,83**	F_2
TAK167/9	44	16	0,09	F_2

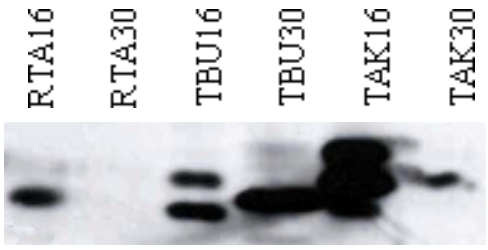

Figure 13. Western blot analysis of scFv in protein extracts of the seeds from F_3 generation of transgenic *Arabidopsis* plants.

RTA16 – cytoplasmic expression of anti-Bl scFv A16 under CaMV 35S promoter; **RTA30** – cytoplasmic expression of anti-Bl scFv A30 under CaMV 35S promoter; **TBU16** – ER expression anti-Bl scFv A16 under USP promoter; **TBU30** – ER expression of anti-Bl scFv A30 under USP promoter; **TAK16** - ER expression anti-Bl scFv A16 under LeB4 promoter; **TAK30** - ER expression anti-Bl scFv A30 under LeB4 promoter.

3.5. ELISA of protein extract from the seeds

To prove the functionality of expressed antibodies in the seeds, ELISA was performed with the seed protein extract. *Arabidopsis* seeds were used from the F_3 generation of the homozygous lines. Protein seed extract was analysed as described in **2.2.5.** On Figure 14 the results of ELISA test of protein seed extract from several lines of transgenic plants are presented. Expressed anti-Bl scFv in the seeds specifically bind to BRs in all of the lines except RTA30. This is a strong evidence of anti-Bl scFv functionality expressed in the seeds. Lines TAK1673 and TAK16152 have the highest ELISA values, TAK1691, TBU1648, TBU16104 slightly lower. Based on the results of the western blotting, TAK16 plants show the highest antibody levels. The ELISA test indicated the highest anti-Bl scFv activity in these plants. TBU16 plants have slightly lower anti-Bl activity and the values of another lines were even less significant. Thus, the high antibody levels detected refer to high anti-Bl scFv activity. Consequently, the transgenic lines with high ELISA values (TAK1673, TAK1691, TAK16152, TBU1684, TBU16104) were selected for further work.

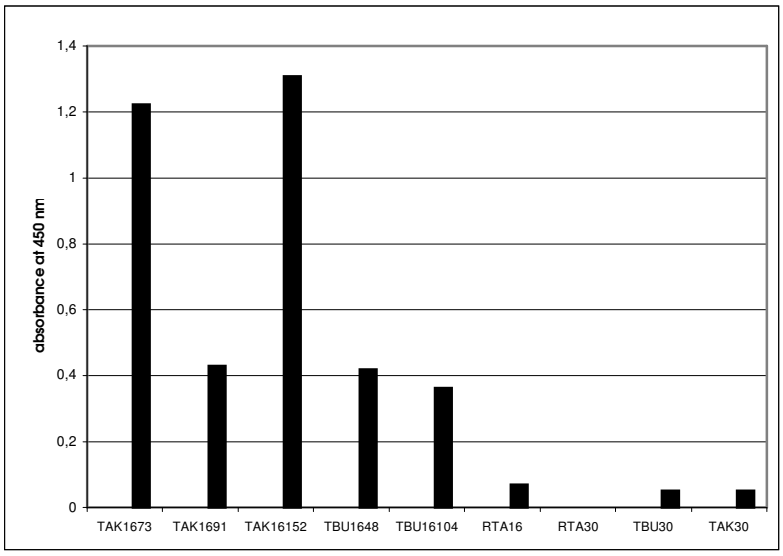

Figure 14. ELISA of protein seed extract from transgenic lines.

TAK1673, TAK1691, TAK16152 – independent transgenic lines of *Arabidopsis* plants with ER expression of anty-Bl scFvA16 under LeB4 promoter; **TBU1648, TBU16104** – independent transgenic lines of *Arabidopsis* plants with ER expression of anty-Bl scFvA16 under USP promoter; **RTA16** - cytoplasmic expression of anti-Bl scFv A16 under CaMV 35S promoter; **RTA30** - cytoplasmic expression of anti-Bl scFv A30 under CaMV 35S promoter; **TBU30** - ER expression of anti-Bl scFv A30 under USP promoter; **TAK30** - ER expression anti-Bl scFv A30 under LeB4 promoter.

3.6. Antibody expression during seed development.

It is known that USP and LeB4 promoters are active in seeds. In tobacco plants USP promoter is active in the first days of seed development (Fiedler et al., 1997) and LeB4 promoter is activated later (Phillips et al., 1997). The expression activities were estimated by measurement of anti-ABA antibodies accumulation in developing transgenic tobacco seeds. The scFv proteins expressed under LeB4 promoter control first could be detected around 14-16 days after pollination (DAP). In USP promoter plants, antibody proteins were already detected 10 DAP.

To investigate the expression of anti-Bl scFv mediated by the USP and LeB4 promoters in the *Arabidopsis* seeds and the dynamic of their presence we measured the anti-Bl scFv accumulation during the seed development in TAK16 and TBU16 transgenic plants. As the live cycle of *Arabidopsis* plants is much shorter then live of tobacco plants, we expected appearance of promoters activities earlier as it was found in tobacco. According to our expectations, in the TBU16 plants expressing anti-Bl scFv under USP promoter control, the anti-Bl presence can be detected on 5^{th} (DAP). It is expressed at a high level from 8^{th} day up to 14^{th} day of development and than the scFv content decreases. In TAK16 plants containing anti-Bl scFv under LeB4 promoter control, the anti-Bl protein appears on the 8^{th} DAP. It is expressed in high level from day 9 and stays at a permanently high level of expression until the seed maturation (Figure 15).

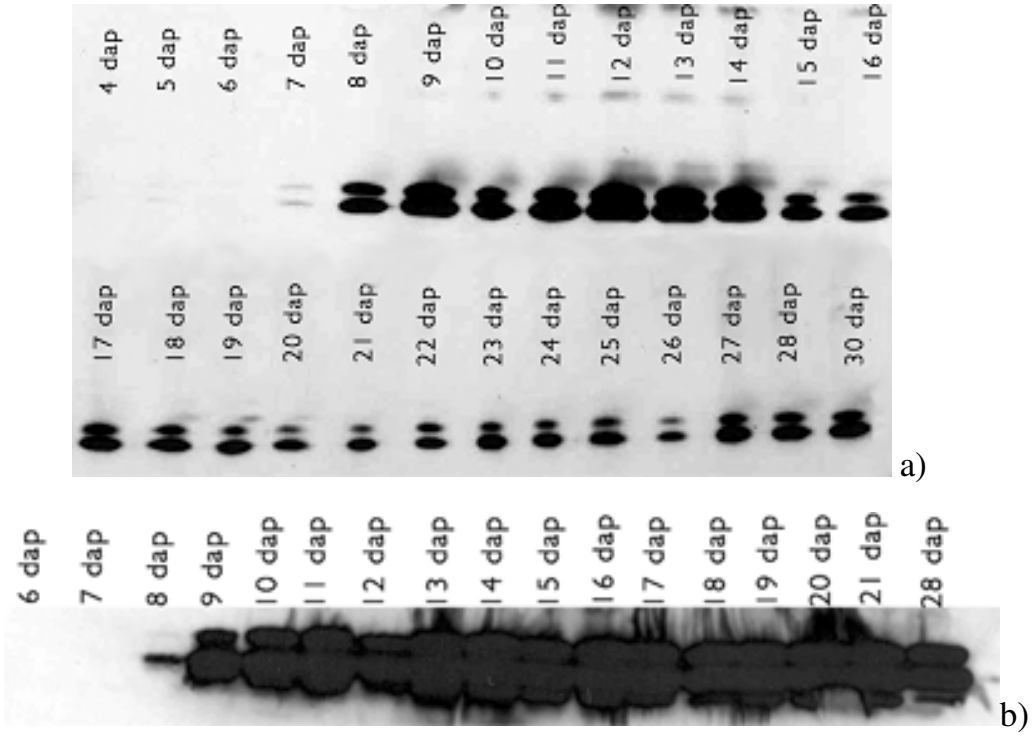

Figure 15. Western blot analysis of anti-Bl expression under control of USP (a) and LeB4 (b) promoters during the seed development. **DAP** - days after pollination

3.7. Ultrastructural changes in transgenic *Arabidopsis* seeds.

Developing seeds of flowering plants synthesize large quantities of nutritive reserves and developmental signals that will be used by the young seedling after germination. Microscopic examination of mature seed shows that all of the cells are filled with the reserves which are used in germination of the seedling which grows dependent on them up to achievement of access to some other nutrient supply. Seeds contain sufficient mineral elements for seedling growth. Phosphorous are stored as phytate (myo-inositol-1,2,3,4,5,6,-hexakisphosphate, InsP6). Phytin, the magnesium, potassium and calcium salt of myo-inositol hexaphosphoric acid, is considered to be a major storage form of phosphorous. This compound is an important anti-nutritional factor, due to its ability to complex essential micro-nutrients e.g. potassium, magnesium, iron, and zinc (Bentsink et al., 2003). It is generally assumed that the major role of InsP6 in plants to act as a storage form of Pi and probably also for cations (Bentsink et al., 2003).

Mature seeds of transgenic *Arabidopsis* plants with anti-Bl scFv A16 expression were analysed and compared to wild type plants using electron microscopy. On Figure 16 is represented result of ultrastructural examinations of the mature seeds of transgenic lines and wild type plants. Dark portion represents protein that is being stored in the protein bodies (PBs). The PBs of mature seed are full. From the picture is visible the slight changes of the PBs shape, size, and content. In wild type plants PBs have round shape close to sphere and they are similar in size. In transgenic lines protein bodies occur in bigger or smaller amount. The shape can be far from the

sphere and size is very much variable. Insight of protein storage vacuoles (PSV) are visible white crystals of phytate (InsP6). Phytin is inherently electron-dense (Lott, 1975), probably because the high concentration of associated metallic cations, and it becomes virtually electron-opaque. The InsP6 appear significantly more often and it is more dens insight of PB in the seeds of transgenic lines than in wild type plants.

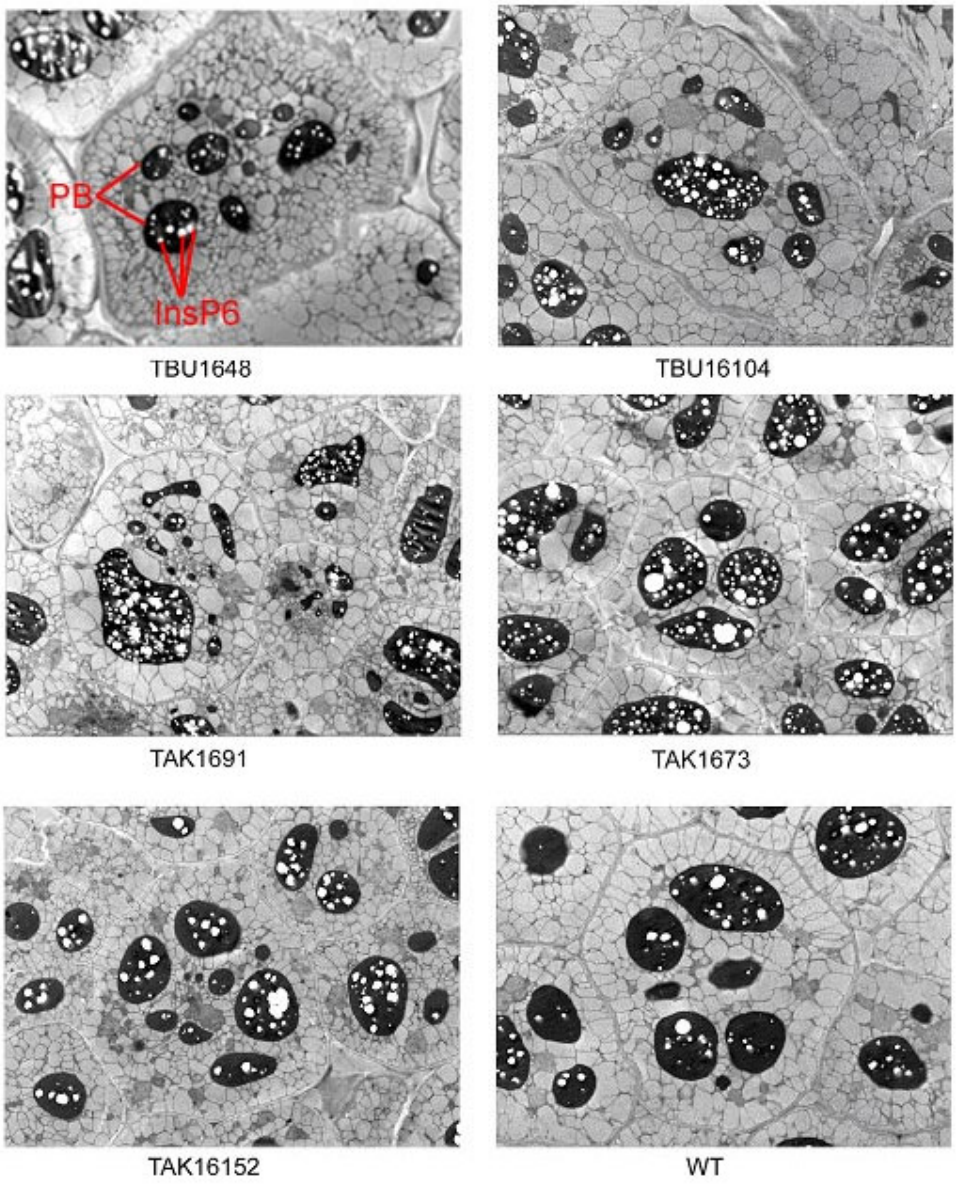

Figure 16. Ultrastructural alterations in the seeds cells of transgenic plants.

Numerous phytate (**IncP6**) crystals can be seen inside the protein body (**PB**). Transgenic lines – **TBU1648, TBU16104, TAK1691, TAK1673, TAK16152**; wild type - **WT**. Images were taken with a CCD camera under Zeiss 902 electron microscope magnification.

3.8 Developmental changes caused by the expression of anti-Bl scFv antibodies

3.8.1 Growth *in vitro*

It has been shown recently, that BRs have a promoter effect on seed germination (Sieber et al., 2001). Since in the immunomodulated plants the hormone function is masked, a germination delay was expected. The differences between transgenic and wild type *Arabidopsis* plants were observed on the second day of germination *in vitro* on Murashige-Skoog medium (Figure 17). Roots of transgenic seedlings from both TAK and TBU lines remained significantly shorter, and the cotyledons were still closed in the seed coat compared to the wild type plants. With the application of 50nM 24-epibrassinolinde to the medium, the germination of transgenic lines could only be partly rescued. On the TBU plants the germination delay is slightly more enhanced then on TAK even if scFv expression under USP promoter is lower. This observation might refer to a more pronounced role of BRs in the early stage of seed development, since the anti-BL protein is expressed earlier in TBU plants (Figure 15).

The developmental delay of the transgenic seedlings can be followed up to the 10^{th} day after germination. At the day 6 of growth, root lengths of the different lines were measured (Figure 18). The roots of transgenic seedlings were still in general 30-40% shorter compared to wild type (Figure 19). Around the 10^{th} day after germination the developmental differences disappear. At this time point the antibodies are not produced any more, thus, with the active BRs the plants overcome the initial growth delay.

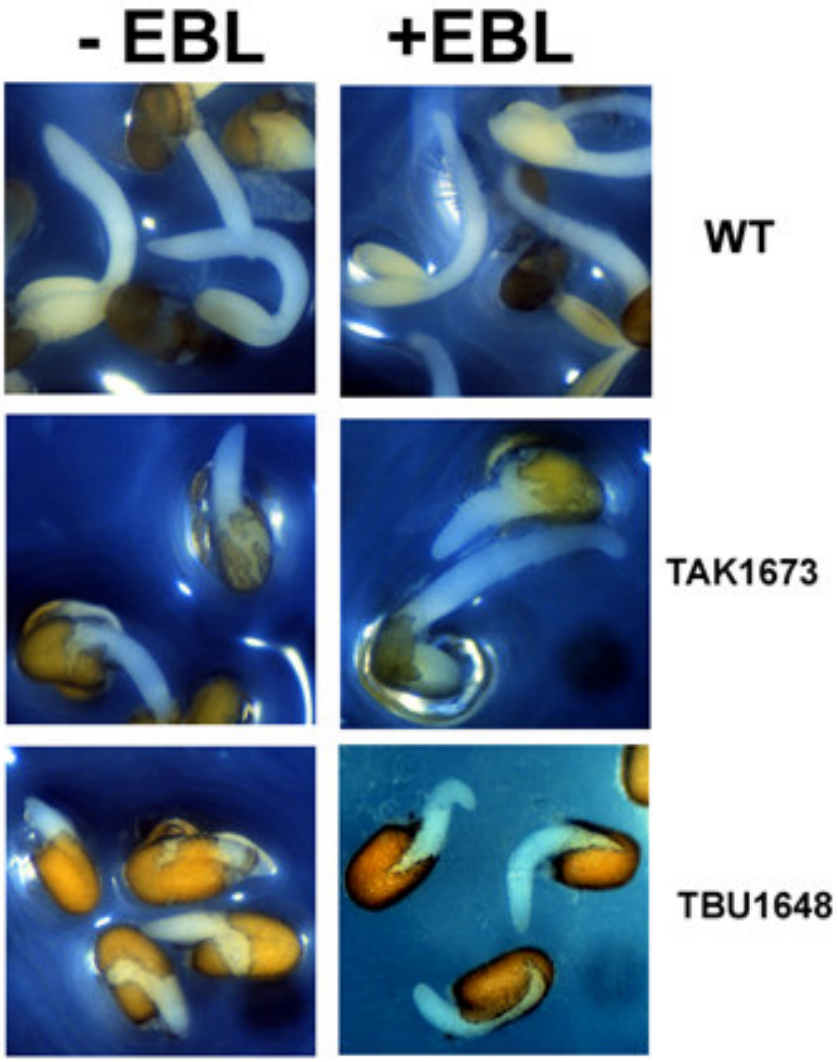

Figure 17. Alterations in seed germination.

Germination experiment was done with the three repetition. Changes were observed on 100 seeds each time. Transgenic (**TAK1673 –** ER expression of anti-BL scFv A16 under LeB4 promoter, **TBU1648 –** ER expression of anti-Bl scFv A16 under USP promoter) and WT plants with application of 50nM of EBL to the MS medium (**+EBL**) and without it (**-EBL**). Photos are taken after 2 days of growth on MS medium *in vitro* under binocular magnification.

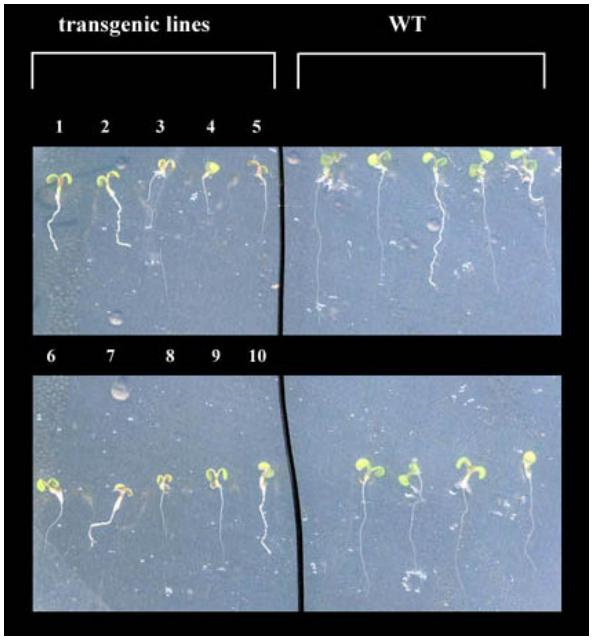

Figure 18. Root development of *Arabidopsis* plants after 6th day of growth.

Seeds were placed and grown up on one Petri dish. Experiments were done three times. Photos are taken by computer scanning. **1-5** – transgenic plants of TAK16152 line (ER expression of anti-Bl scFv A16 under LeB4 promoter); **6-10** – transgenic plants of TBU1648 line (ER expression of scFv anti- Bl scFv 16 under USP promoter). **WT** – wild type plants.

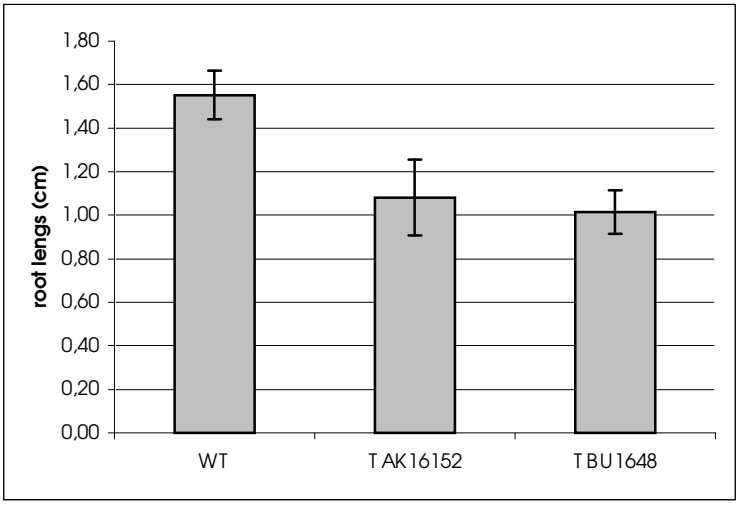

Figure 19. Roots lengths of 6th days old *Arabidopsis* plants.

Average data of 20 individual plants. **WT** – wild type plants; **TAK16152** – ER expression of anti-Bl scFv under LeB4 promoter; **TBU1648** – ER expression of anti-Bl scFv under USP promoter.

3.8.2. Growth *in vivo*

The developmental delay of the transgenic seedlings can be prolonged by transferring germinated seeds to the soil. At the day 6th of growth *in vitro* plants were transferred to the soil and cultivated in the growth chamber at 22°C with 6000 lux of white light for 8 hours in a day. After 15 days of growth size of different transgenic lines was estimated and compared to wild type. The transgenic plants of TAK 16 line were generally smaller and preserved the developmental delay after their transferring to the soil. The plants of TBU16 line after 15 days of growth in the soil overcame the initial retardation, moreover, in comparison to wild type plants they were better developed (in general 2-3 leaves more). The RTA16 lines were growing similarly to the wild type (Figure 20).

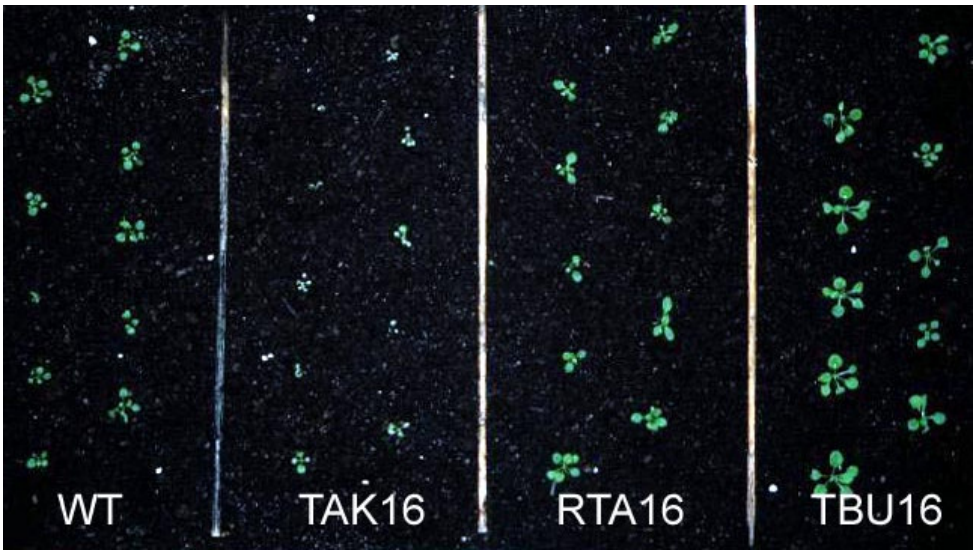

Figure 20. Growth of transgenic plants *in vivo*.

Plants were cultivated in the growth chambers at 22°C with short day (8 h) light. Pictures were taken after 15 days of transfer from *in vitro in vivo* by photo camera. **WT** – wild type plants; **TAK16152** – ER expression of anti-Bl scFv under LeB4 promoter; **TBU1648** – ER expression of anti-Bl scFv under USP promoter; **RTA16** – cytosol expression of anti-Bl scFv under CaMV 35S promoter.

3.9. Gene expression analysis during seed germination using Affymetrix GeneChip.

A GeneChip® probe array is a tool used to monitor gene expressions for thousands of transcripts, and allows highly reproducible, parallel quantification of gene expression levels. The GeneChip Arabidopsis ATH1 Genome Array, designed in collaboration with The Institute of Genome Research (TIGR), contains more than 22500 probe sets representing approximately 24000 genes. The array is based on information from the international Arabidopsis sequencing project that was formally completed in December, 2000. In parallel and subsequent to the genome' s completion, TIGR reannotated the entire genome in a project funded by the National Science Foundation, and the resulting data was used in the design of this array. Data from the TIGR database (ATH1-121501) hosted in the NetAffx™ Analysis Center (http://www.affymetrix.com/analysis/index.affx).

Wild-type and TAK1673 transgenic *Arabidopsis* seeds were grown *in vitro* on MS medium. The RNA for making the hybridisation probes was isolated from large pool of germinated seeds after 6 days of growth *in vitro*. The obtained data were analysed by means of the Microarray Suite (version 4.0, Affymetrix) software. To calculate absolute call metrics, the program includes three metrics: positive fraction, positive/negative ration, and log average ration. They serve to estimate cross hybridisation and the signal specificity. If certain critical values are exceeded, a gene call is present. Only present genes were used for comparison and with a signal log ration more then 1 were selected like genes with significantly changed expression profile. Wild type and transgenic plants were compared by means of Affymetrix Genome Array hybridisations. Hundred six genes were displayed "increase" (s tronger expression in transgenic plants) and two hundred fifty genes displayed "decrease" (stronger expression in wild type plants). The genes with known or proposed functions were sorted and presented in table 5.

Table 5. Genes differentially expressed in *Arabidopsis* germinating seeds.

Seeds of wild type plants and transgenic TAK1673 plants were harvested after 6 days of grown. **Signal** - numbers indicate the hybridisation signal intensities; **SLR** – signal log ratio (a change in expression level for a transcript). This change is expressed as the $\log_2$ ratio. A $\log_2$ ratio of 1 is the same as a fold change of 2. - genes down-regulated in transgenic plants; - genes up-regulated in transgenic lines.

Signal	SLR	Descriptions
26,4	2,1	late embryogenesis abundant M17 protein identical to GB:AF076979
55,2	1,2	late embryogenesis-abundant protein, putative similar to late embryogenesis-abundant protein GI:17828 from [Brassica napus];supported by full-length cDNA: Ceres:35745.
113,6	1,0	similar to late embryogenesis abundant proteins
80,9	1,2	late embryogenis abundant protein, putative similar to late embryogenis abundant protein 5 GI:2981167 from [Nicotiana tabacum];supported by full-length cDNA: Ceres:96540.
91,9	5,9	NWMU2 - 2S albumin 2 precursor
142,9	4,8	NWMU3 - 2S albumin 3 precursor
172,5	4,4	NWMU1 - 2S albumin 1 precursor; supported by cDNA: gi_13899092_gb_AF370541.1_AF370541
87,2	3,5	NWMU4 - 2S albumin 4 precursor; supported by cDNA: gi_15450628_gb_AY052682.1_
132,4	3,4	putative cruciferin 12S seed storage protein identical to 12S seed storage protein, gi|808937
221,5	2,3	12S cruciferin seed storage protein
45,7	1,3	2S storage protein-like
192,4	1,0	legumin-like protein
71,1	2,5	heat shock protein 17.6A
213,8	1,9	heat shock protein 70 identical to heat shock protein 70 GB:CAA05547 GI:3962377 [Arabidopsis thaliana]; supported by cDNA: gi_15809831_gb_AY054183.1_
70,9	1,9	heat shock protein, putative similar to heat shock protein GI:19617 from [Medicago sativa];supported by full-length cDNA: Ceres:32795.
13,8	1,8	heat shock transcription factor-like protein
72,1	1,4	low-molecular-weight heat shock protein - like cytosolic class I small heat-shock protein HSP17.5, Castanea sativa, EMBL:CSA9880
103,6	1,3	heat-shock protein; supported by cDNA: gi_166769_gb_M62984.1_ATHHSP83
60,6	1,1	heat shock protein 101 (HSP101) identical to heat shock protein 101 GI:6715468 GB:AAF26423 from [Arabidopsis thaliana]
80,8	1,0	putative heat shock protein; supported by full-length cDNA: Ceres:25528.
50,6	1,0	mitochondrial heat shock 22 kd protein-like; supported by full-length cDNA: Ceres: 268536.
1764	2,0	ribosomal protein L22
2107	1,8	ribosomal protein L16

Signal	SLR	Descriptions
1478	1,7	ribosomal protein S8
612,1	1,7	ribosomal protein L14
3070	1,6	ribosomal protein S11
2102	1,5	ribosomal protein L36
2495	1,5	ribosomal protein S3
2139	1,5	ribosomal protein S19
146	1,1	putative 60s ribosomal protein L37 almost identical to GB:Q43292;supported by full-length cDNA: Ceres:11912.
260,7	1,1	ribosomal protein L33 - like ribosomal protein L33, Rickettsia prowazekii, PIR:E71650
1562	1,0	ribosomal protein ; supported by full-length cDNA: Ceres: 18153.
2087	1,0	ribosomal protein S25 ribosomal protein S25, Lycopersicon esculentum, PIR2:S40089;supported by full-length cDNA: Ceres:15801.
623,5	1,0	ribosomal protein L35 - like ribosomal protein L35- cytosolic, Arabidopsis thaliana, PIR:T00549;supported by full-length cDNA: Ceres:14525.
1186	1,0	ribosomal protein L27, putative similar to 60S RIBOSOMAL PROTEIN L27 GB:P41101 from [Solanum tuberosum];supported by full-length cDNA: Ceres:25631.
152,9	1,0	ribosomal protein, putative similar to ribosomal protein L35a GI:57118 from [Rattus norvegicus]; supported by full-length cDNA: Ceres: 2778.
1674	1,0	putative ribosomal protein similar to ribosomal protein L37 GB:BAA04888 from [Homo sapiens];supported by full-length cDNA: Ceres:14710.
657,1	1,0	ribosomal protein L29, putative similar to 60S ribosomal protein L29 GB:P25886 from [Rattus norvegicus]
511,4	1,0	putative ribosomal protein similar to ribosomal protein L33B GB:NP_014877 from [Saccharomyces cerevisiae]; supported by full-length cDNA: Ceres: 20141.
8,5	2,3	putative translation initiation factor IF2
12,4	1,6	homeodomain transcription factor (ATHB-4)
34,4	1,5	putative AP2 domain transcription factor
20,2	1,4	putative heat shock transcription factor
117,8	1,1	Prematurely terminated mRNA decay factor-like protein
594,8	1,1	RNA polymerase beta subunit-1
23,9	1,0	myb-related protein M4; supported by full-length cDNA: Ceres:33333.
134,2	1,0	putative transcription factor Arabidopsis thaliana mRNA for GATA transcription factor 3, PID:e1254739
208,3	1,0	DNA-directed RNA polymerase (EC 2.7.7.6) II largest chain
12,5	1,4	myb protein(MYB27) contains myb DNA binding domain:- PF00249
673,2	1,3	putative protein similar to transcriptional regulator;supported by full-length cDNA: Ceres:9218.
66,3	1,2	unknown protein contains similarity to DNA dependent reverse transcriptase GI:2920563 from [Spraguea lophii]
116,3	1,1	putative fatty acid elongase; supported by full-length cDNA: Ceres:115769.
23,3	2,1	subtilisin proteinase - like subtilisin-like proteinase ag12, Alnus glutinosa, PIR2:S52769; supported by cDNA: gi_16649028_gb_AY059884.1_
92,5	1,4	subtilisin-like serine protease similar to subtilisin-type protease precursor GI:14150446 from [Glycine max];supported by full-length cDNA: Ceres:3907.
40,5	1,1	subtilisin-like serine protease contains similarity to prepro-cucumisin GI:807698 from [Cucumis melo]
699,1	1,1	ATP-dependent Clp protease ATP-binding subunit (ClpD), ERD1 protein precursor identical to ERD1 protein GI:497629, SP:P42762 from [Arabidopsis thaliana]
284,6	1,0	subtilisin-like serine protease, putative contains similarity to cucumisin-like serine protease GI:3176874 from [Arabidopsis thaliana]
107,5	1,0	putative glucose acyltransferase similar to glucose acyltransferase GB:AAD01263 [Solanum berthaultii]; also similar to serine carboxypeptidase I GB:P37890 [Oryza sativa]; supported by full-length cDNA: Ceres: 94163.
513,7	1,0	FtsH protease, putative similar to zinc dependent protease GI:7650138 from [Arabidopsis thaliana]
34,5	1,6	pyruvate kinase -like protein various pyruvate kinases from procaryotes
19,1	1,5	receptor protein kinase like protein lectin receptor-like serine/threonine kinase lecRK1, Arabidopsis thaliana, PIR2:S68589
32,5	1,3	leucine-rich receptor-like protein kinase - like protein leucine-rich receptor-like protein kinase LRPKm1, Malus domestica, EMBL:AF053127
34,7	1,2	hypothetical protein contains similarity to wall-associated kinase 2 GI:4826399 from [Arabidopsis thaliana]
188,4	1,1	phosphatidylinositol 4-kinase (emb|CAB37928.1)
69,9	1,1	MAP kinase kinase 4 (ATMKK4) identical to MAP kinase kinase 4 [Arabidopsis thaliana]; supported by cDNA: gi_13265419_gb_AF324667.2_AF324667
27,1	1,1	KI domain interacting kinase 1 -like protein KI domain interacting kinase 1 - Zea mays,PID:g2735017
188,4	1,1	phosphatidylinositol 4-kinase (emb|CAB37928.1)
34	1,0	protein kinase, putative contains Pfam profile: PF00069: Eukaryotic protein kinase domain

Signal	SLR	Descriptions
25,1	1,0	putative protein kinase similar to protein kinase homolog GB:AAC78477 from [Arabidopsis thaliana];supported by full-length cDNA: Ceres:96699.
31	1,0	putative protein different MAP kinases
6,5	2,8	receptor-like kinase, putative similar to receptor-like kinase GI:5523856 from [Hordeum vulgare]
52,8	1,7	serine/threonine kinase-like protein receptor-like protein kinase RLK3 - Arabidopsis thaliana,PID:e1363211
21,3	1,4	receptor-like protein kinase receptor like protein kinase - Arabidopsis thaliana,PID:e1188577
146,8	2,7	putative glutathione transferase Second of three repeated putative glutathione transferases. 72% identical to glutathione transferase [Arabidopsis thaliana] (gi|4006934). Location of ests 191A10T7 (gb|R90188) and 171N13T7 (gb|R65532)
110,9	2,1	putative isocitrate lyase similar to GB:P25248 from [Brassica napus]
21,4	2,0	putative glucosyltransferase; supported by full-length cDNA: Ceres:153418.
741,2	1,9	beta-glucosidase-like protein several beta-glucosidases - different species; supported by cDNA: gi_10834547_gb_AF159376.1_AF159376
39,5	1,7	amino acid permease 6 (emb CAA65051.1)
12,4	1,7	putative caffeic acid 3-O-methyltransferase similar to caffeic acid 3-O-methyltransferase GB:O23760 [Clarkia breweri]
405,1	1,6	putative beta-alanine-pyruvate aminotransferase; supported by full-length cDNA: Ceres:147543.
35,4	1,5	anthocyanidin-3-glucoside rhamnosyltransferase-like
58,9	1,5	O-methyltransferase, putative similar to GB:AAF28353 from [Fragaria x ananassa]
339,9	1,5	NADH dehydrogenase subunit 3 Protein sequence is in conflict with the conceptual translation
112,4	1,4	putative sucrose synthetase similar to several plant sugar synthetases similar to P. sativum second sugar synthetase, GenBank accession number AJ001071 similar to beet sucrose synthetase (EC 2.4.1.13), GenBank accession number S71494; supported by cDNA: gi_15293134_gb_AY051001.1_
34,5	1,4	putative ATPase similar to GB:AAF28353 from [Fragaria x ananassa]
51	1,4	putative ATP-dependent RNA helicase
12	1,4	putative reticuline oxidase-like protein similar to GB:P30986 from [Eschscholzia californica] (berberine bridge-forming enzyme), ESTs gb|F19886, gb|Z30784 and gb|Z30785 come from this gene
518,6	1,3	unknown protein similar to L-allo-threonine aldolase (D87890); similar to ESTs gb|R30517, gb|T42772, gb|R90493, and gb|R90493;supported by full-length cDNA: Ceres:271327.
102,1	1,3	putative L-asparaginase similar to L-ASPARAGINASE GB:P30364from [Lupinus angustifolius];supported by full-length cDNA: Ceres:21689.
32,3	1,3	cyclopropane-fatty-acyl-phospholipid synthase, putative similar to cyclopropane-fatty-acyl-phospholipid synthase GB:P30010 [Escherichia coli]
49,7	1,3	NADH dehydrogenase subunit 5 (nad5) (trans-splicing part 1 of 2)
2039	1,3	glutamine-dependent asparagine synthetase; supported by cDNA: gi_16930406_gb_AF419557.1_AF419557
518,6	1,3	unknown protein similar to L-allo-threonine aldolase (D87890); similar to ESTs gb|R30517, gb|T42772, gb|R90493, and gb|R90493;supported by full-length cDNA: Ceres:271327.
102,1	1,3	putative L-asparaginase similar to L-ASPARAGINASE GB:P30364from [Lupinus angustifolius];supported by full-length cDNA: Ceres:21689.
42,7	1,3	hypothetical protein contains similarity to endonuclease III; supported by cDNA: gi_15294153_gb_AF410268.1_AF410268
102,1	1,3	putative L-asparaginase similar to L-ASPARAGINASE GB:P30364from [Lupinus angustifolius];supported by full-length cDNA: Ceres:21689.
113,5	1,2	putative DNA2-NAM7 helicase family protein
109	1,2	basic chitinase identical to basic chitinase GB:AAA32769 GI:166666 [Arabidopsis thaliana] (Plant Physiol. 93, 907-914 (1990)); supported by cDNA: gi_15451095_gb_AY054628.1_
169,4	1,2	E2, ubiquitin-conjugating enzyme, putative
89,4	1,2	delta 9 desaturase identical to delta 9 desaturase GB:BAA25180 GI:2970034 from [Arabidopsis thaliana]; supported by full-length cDNA: Ceres: 100525.
925	1,2	beta-fructosidase identical to beta-fructosidase GI:1871503 from [Arabidopsis thaliana]; supported by cDNA: gi_15028118_gb_AY046009.1_
147	1,2	putative galactinol synthase; supported by full-length cDNA: Ceres:124236.
51	1,2	putative beta-ketoacyl-CoA synthase Strong similarity to beta-keto-Coa synthase gb|U37088 from Simmondsia chinensis
37,3	1,2	peroxidase, putative similar to peroxidase GB:P80679 from [Armoracia rusticana]
292,3	1,1	NADH dehydrogenase subunit 4
181,7	1,1	glutathione transferase, putative similar to glutathione transferase GI:2853219 from [Carica papaya];supported by full-length cDNA: Ceres:252874.
44,3	1,1	CTP synthase like protein CTP synthase, Methanococcus jannaschii,PIR2:E64446
116,7	1,1	lipoxygenase, putative similar to lipoxygenase GI:1407704 from [Solanum tuberosum]; supported by cDNA: gi_289202_gb_L04637.1_ATHLIPOXY
31,5	1,1	acetyl-CoA carboxylase, putative similar to GI:1100253 from [Arabidopsis thaliana]
89,8	1,1	kinesin-like protein
824,8	1,1	lysine-ketoglutarate reductase/saccharopine; supported by cDNA: gi_2052507
85,4	1,1	beta-glucosidase, putative similar to beta-glucosidase GB:AAB64244 from [Arabidopsis thaliana], (Plant Mol. Biol. 34 (1), 57-68 (1997))

Signal	SLR	Descriptions
365,3	1,1	methionine/cystathionine gamma lyase, putative similar to methionine gamma-lyase GB:CAA04124.1 GI:2330885 from [Trichomonas vaginalis]; supported by cDNA: gi_15450931_gb_AY054546.1_
44,3	1,1	CTP synthase like protein CTP synthase, Methanococcus jannaschii,PIR2:E64446
116,8	1,1	putative glucose-6-phosphate dehydrogenase Similar to gi|2276344, gi|2829880, gi|2352919 and others; Location of EST gb|N37552, gc exon splice site at 20574 is based on protein alignment, and is not confirmed experimentally; supported by cDNA: gi_15810386_gb_AY056232.1_
58,9	1,1	unknown protein contains similarity to alternative NADH-dehydrogenase GI:3718005 from [Yarrowia lipolytica];supported by full-length cDNA: Ceres:114420.
58,9	1,1	unknown protein contains similarity to alternative NADH-dehydrogenase GI:3718005 from [Yarrowia lipolytica];supported by full-length cDNA: Ceres:114420.
59,8	1,1	putative protein strictosidine synthase (EC 4.3.3.2) - Rauvolfia mannii (fragment); supported by cDNA: gi_13877836_gb_AF370181.1_AF370181
58,9	1,1	unknown protein contains similarity to alternative NADH-dehydrogenase GI:3718005 from [Yarrowia lipolytica];supported by full-length cDNA: Ceres:114420.
296,3	1,1	putative glucan synthase
1694	1,0	NADH dehydrogenase ND1
262,4	1,0	serine acetyltransferase identical to GB:CAA84371 from [Arabidopsis thaliana] (Eur. J. Biochem. 227 (1-2), 500-509 (1995)); supported by cDNA: gi_926938_gb_L42212.1_ATHSAT1G
804	1,0	fructose-2,6-bisphosphatase, putative similar to fructose-2,6-bisphosphatase GI:8572069 from [Arabidopsis thaliana]; supported by cDNA: gi_13096097_gb_AF190739.2_AF190739
870,8	1,0	carboxytransferase beta subunit
224	1,0	glucosyltransferase like protein ; supported by cDNA: gi_2149126_gb_U81293.1_ATU81293
23,4	1,0	peroxidase C2 precursor like protein peroxidase (EC 1.11.1.7) C2 precursor - Armoracia rusticana,PID:d1014846
299,6	1,0	quinone oxidoreductase -like protein probable quinone oxidoreductase (EC 1.6.5.5) P1, Arabidopsis thaliana, PIR:S57611
92,3	1,0	endoxyloglucan transferase (gb|AAD45127.1); supported by cDNA: gi_1244751_gb_U43485.1_ATU43485
87,1	1,0	N-hydroxycinnamoyl/benzoyltransferase-like protein; supported by cDNA: gi_14334525_gb_AY034954.1_
14,4	1,0	similar to flavin-binding monooxygenase (Z71258); similar to ESTs gb|R30018, gb|T23015, and gb|T88100 contains similarity to flavin-containing monooxygenase 2 GB:AAD56413 GI:5923916 from [Mus musculus]
33,9	1,0	acyl CoA reductase -like protein acyl CoA reductase, Simmondsia chinensis, EMBL:AF149917
113,4	1,0	putative peroxidase Strong similarity to Arabidopsis peroxidase ATPEROX7A (gb|X98321);supported by full-length cDNA: Ceres:114862.
314,2	1,0	putative indole-3-acetate beta-glucosyltransferase similar to indole-3-acetate beta-glucosyltransferase GB:AAD32293
503,8	1,0	xyloglucan endo-1,4-beta-D-glucanase-like protein xyloglucan endo-1,4-beta-D-glucanase (EC 3.2.1.-) XTR-3 - Arabidopsis thaliana,PIR2:S71222; supported by cDNA: gi_15215689_gb_AY050373.1_
455,4	1,0	hypothetical protein contains similarity to ubiquitin protein ligase GI:2827198 from [Mus musculus]
351,6	1,5	lipoxygenase AtLOX2 ; supported by cDNA: gi_431257_gb_L23968.1_ATHATLO
52,1	1,5	putative amino-cyclopropane-carboxylic acid oxidase (ACC oxidase) Strong similarity to amino-cyclopropane-carboxylic acid oxidase gb|L27664 from Brassica napus. ESTs gb|Z48548 and gb|Z48549 come from this gene; supported by cDNA: gi_15450652_gb_AY052694.1_
157,4	1,4	2,4-D inducible glutathione S-transferase, putative similar to 2,4-D inducible glutathione S-transferase GI:2920666 from [Glycine max];supported by full-length cDNA: Ceres:30680.
40,3	1,3	dynein light chain, putative similar to dynein light chain 1, cytoplasmic SP:Q15701 [Homo sapiens]
113,9	1,3	phosphoethanolamine N-methyltransferase, putative similar to GI:7407189 from [Spinacia oleracea]
188,9	1,3	zinc finger and C2 domain protein, putative similar to zinc finger and C2 domain protein GI:9957238 from [Arabidopsis thaliana]; supported by full-length cDNA: Ceres: 148076.
36,3	1,2	putative endochitinase
71,8	1,2	glucose-6-phosphate 1-dehydrogenase strongly similar to GB:S71245, location of EST gb|Z35060 and gb|T04591
120	1,1	glutaredoxin -like protein glutaredoxin, castor bean, PIR:S54825
62,6	1,0	catechol O-methyltransferase, putative similar to catechol O-methyltransferase GI:4808524 from [Thalictrum tuberosum]
62,6	2,0	putative squamosa-promoter binding protein
170,1	1,9	putative protein similarity to low-temperature-induced protein 65, Arabidopsis thaliana, PIR2:S30153~contains EST gb:W43419, W4351200
3250	1,8	PSI P700 apoprotein A1
25,9	1,7	unknown protein contains similarity to DNA-binding protein zyxin GB:X99063 GI:1430882 from [Mus musculus]
33,4	1,7	hypothetical protein contains Cys3His zinc finger domain
24,4	1,5	putative protein ethylene-responsive element binding protein homolog, Stylosanthes hamata, U91857
42,3	1,5	putative chlorophyll A-B binding protein similar to chlorophyll A-B binding protein 151 precursor (LHCP) GB:P27518 from [Gossypium hirsutum];supported by full-length cDNA: Ceres:7700.
143,2	1,5	putative ABC transporter contains Pfam profile: PF00005 ABC transporter
220,6	1,5	ammonium transporter, puitative similar to ammonium transporter GI:5880357 from [Arabidopsis thaliana]; supported by cDNA: gi_4324713_gb_AF110771.1_AF110771
281,2	1,5	MATE efflux family protein, putative contains TIGRfam profile: TIGR00797: MATE efflux family protein

Signal	SLR	Descriptions
32	1,5	putative glycine-rich RNA-binding protein
223,6	1,4	unknown protein contains a domain related to disease resistance genes and a proline-rich domain
146,9	1,4	putative protein nucleoid DNA-binding protein cnd41, chloroplast, common tobacco, PIR:T01996; supported by cDNA: gi_15809849_gb_AY054192.1_
196,4	1,3	putative protein mRNA of unknown function, Homo sapiens, EMBL:HS598F21A_1
52,6	1,3	putative protein RXF26, Arabidopsis thaliana, EMBL:AB008020
21	1,3	putative disease resistance protein Cf-4 ,Lycopersicon hirsutum, gb:AJ002235
35,3	1,3	unknown protein contains similarity to calcium-binding protein GB:CAB63264 GI:6580549 from [Lotus japonicus]; supported by cDNA: gi_13605536_gb_AF361594.1_AF361594
25,5	1,3	zinc finger protein, putative similar to finger protein pcp1 GB:S48856 from [Solanum tuberosum]; supported by cDNA: gi_13605632_gb_AF361797.1_AF361797
93,1	1,3	BCS1 protein-like protein Homo sapiens h-bcs1 (BCS1) mRNA, nuclear gene encoding mitochondrial protein which is involved in the expression of functional mitochondrial ubiquinol-cytochrome c reductase complex probably via the control of expression of Rieske iron-sulphur protein, PI
115	1,3	putative ABC transporter related to multi drug resistance proteins and P-glycoproteins
169,5	1,3	auxin response factor - like protein auxin response factor 9, Arabidopsis thaliana, PIR:T08917
3199	1,2	cytochrome B6
231,7	1,2	cytochrome P450
81,4	1,2	disease resistance protein-like
57,9	1,2	FRO1 and FRO2-like protein
51,9	1,2	putative protein leucine zipper-containing protein, tomato, PIR:S21495; supported by cDNA: gi_15215763_gb_AY050411.1_
212,8	1,2	viral resistance protein, putative, 5 partial similar to viral resistance protein GI:7110565 from [Arabidopsis thaliana]
570,7	1,2	putative ABC transporter similar to Guillardia theta ABC transporter, GenBank accession number AF041468; supported by cDNA: gi_15529142_gb_AY052195.1_
26,3	1,1	putative oleosin protein
1355	1,1	PSII 10KDa phosphoprotein
97,8	1,1	myosin-like protein unconventional myosin heavy chain, Zea mays, PIR:A59310
179,5	1,1	proline-rich protein other proline-rich proteins
227,9	1,1	dehydrin RAB18-like protein (sp P30185) ; supported by cDNA: gi_16226664_gb_AF428458.1_AF428458
31,6	1,1	low-temperature-induced 65 kD protein (sp|Q04980)
2817	1,1	P-Protein - like protein P-Protein precursor, Solanum tuberosum, gb:Z99770; supported by cDNA: gi_14596024_gb_AY042800.1_
83,6	1,1	disease resistance protein-like
30,4	1,1	disease resistance protein-like
48,1	1,1	stress-induced protein sti1 -like protein stress-induced protein sti1 -Glycine max,PID:g872116
103,3	1,1	zinc finger protein (PMZ), putative identical to putative zinc finger protein (PMZ) GB:AAD37511 GI:5006473 [Arabidopsis thaliana]
85,1	1,1	n-calpain-1 large subunit, putative similar to GI:882072 from [Gallus gallus] (Biochim. Biophys. Acta 1261 (3), 381-393 (1995))
147,7	1,1	glycine-rich RNA-binding protein grp1a, putative similar to glycine-rich RNA-binding protein grp1a GB:L31374 GI:496232 from [Sinapis alba]
12,6	1,1	ABC transporter, putative similar to ABC transporter GI:9279716 from (Arabidopsis thaliana)
1218	1,1	ferritin 1 precursor;supported by full-length cDNA: Ceres:1100.
69,9	1,1	putative protein non-consensus CG donor splice site at exon 1, GA donor splice site at exon 3, similar to unknown protein (pir||T04268);supported by full-length cDNA: Ceres:32257.
43,3	1,0	putative protein NLS receptor - Oryza sativa, PID:d1032113
66.3	1,0	HD-Zip protein, putative similar to HD-Zip protein GI:2145356 from [Arabidopsis thaliana]; supported by cDNA: gi_16974580_gb_AY060556.1_
115,1	1,0	putative protein membrane-associated salt-inducible protein, Nicotiana tabacum, PIR:T02047
43,2	1,0	putative pumilio/Mpt5 family RNA-binding protein
110	1,0	putative embryo-abundant protein
45,4	1,0	putative protein contains similarity to salt-stress responding gene product; supported by cDNA: gi_14091591_gb_AF369572.1_AF369572
205,6	1,0	putative photomorphogenesis repressor protein
316,5	1,0	F-box protein family, AtFBL6 contains similarity to grr1 GI:2407790 from [Glycine max]
52,8	1,0	nodulin-26 - like protein major intrinsic protein, Oryza sativa, PIR2:S52003;supported by full-length cDNA: Ceres:109513.
67,4	1,0	FCA gamma protein
80,7	1,0	sigma-like factor (emb CAA77213.1)
464,3	1,0	MtN3-like protein; supported by cDNA: gi_3747110_gb_AF095641.1_AF095641
27,4	1,0	lateral root primordia (LRP1)
71,3	1,0	similar to terminal flower; supported by cDNA: gi_4521162_dbj_AB024715.1_AB024715
71,5	1,0	disease resistance protein-like

Signal	SLR	Descriptions
263,4	1,0	disease resistance protein homolog disease resistance protein RPP1-WsB - Arabidopsis thaliana, EMBL:AF098963
70,3	1,0	disease resistance protein; supported by cDNA: gi_15215703_gb_AY050380.1_
54,4	1,0	putative ABC transporter
30,1	1,0	P-glycoprotein, putative similar to P-glycoprotein GB:A42150 from [Arabidopsis thaliana]
50,5	1,0	putative cytochrome P450 similar to GB:Q05047 from [Catharanthus roseus]
33	1,0	putative disease resistance protein similar to Cf-2 disease resistance protein GB:AAC15780 from [Lycopersicon pimpinellifolium]
111,8	1,0	putative phorbol ester / diacylglycerol binding protein Pfam HMM hit: Phorbol esters / diacylglycerol binding domain
45,7	1,0	putative protein amino acid transport protein - Arabidopsis thaliana, EMBL:U39783
628,4	1,0	ABC transporter protein 1-like; supported by cDNA: gi_15912314_gb_AY056435.1_
77,3	1,0	transporter-like protein oligopeptide transporter (LeOPT1) - Lycopersicon esculentum, EMBL:AF016713
119,9	5,8	trypsin inhibitor 2, putative similar to trypsin inhibitor 2 GI:4902896 from [Sinapis alba]
1,9	5,4	CLE7, putative CLAVATA3/ESR-related 7 (CLE7)
39,8	4,7	putative thionin
24,7	3,0	thaumatin-like protein similar to thaumatin GB:AAD55090 [Vitis riparia]; contains Pfam profile: PF00314 Thaumatin family; supported by cDNA: gi_14334527_gb_AY035168.1_
14,9	2,9	embryo-specific protein 1 (ATS1)
63,9	2,5	probable wound-induced protein wound-induced protein, Lycopersicon esculentum, PIR2:S19773;supported by full-length cDNA: Ceres:1831.
36,3	1,7	putative protein PRE87 mRNA, Pinus radiata, AF049069
107,4	1,7	putative cell wall-plasma membrane disconnecting CLCT protein (AIR1A); supported by full-length cDNA: Ceres:20383.
38,4	1,5	putative squamosa-promoter binding protein ;supported by full-length cDNA: Ceres:10375.
93,9	1,4	putative pathogenesis-related protein
79,9	1,3	putative protein hypersensitivity-related hsr201 protein - Nicotiana tabacum,PIR2:T03274
380,9	1,3	thionin Thi2.2; supported by full-length cDNA: Ceres:1523.
200,6	1,3	pEARLI 1-like protein Arabidopsis thaliana pEARLI 1 mRNA, PID:g871780; supported by cDNA: gi_15450470_gb_AY052336.1_
35,6	1,2	oleosin, 18.5K
398,9	1,2	putative lectin similar to lectin SP:P02874 [Onobrychis viciifolia]; contains Pfam profile: PF00139 legume lectins beta domain; supported by cDNA: gi_15809853_gb_AY054194.1_
316,1	1,2	low temperature and salt responsive protein homolog low temperature and salt responsive protein LTI6A - Arabidopsis thaliana,PID:g4039153
113,3	1,1	cytochrome P450
35,5	1,1	putative CCHC-type zinc finger protein contains Pfam profile: PF00098 zinc finger, CCHC class
83,8	1,1	putative thioredoxin similar to thioredoxin GB:S58123 [Arabidopsis thaliana]
676,2	1,1	putative auxin-regulated protein auxin-induced protein X15, Glycine max, PIR2:JQ1097;supported by full-length cDNA: Ceres:10510.
626,1	1,0	pEARLI 1 ; supported by cDNA: gi_871779_gb_L43080.1_ATHPEAR
693,2	1,0	chaperonin CPN10 identical to SP:P34893 from [Arabidopsis thaliana];supported by full-length cDNA: Ceres:26943.

3.10. Genes with altered expression in the transgenic lines.

Semi-quantitative expression studies were performed on one gene *12S* (putative cruciferin 12S seed storage protein identical to 12S seed storage protein, gi808937). Cruciferin (12S globulin) is a large, neutral, oligometric protein synthesized in rapeseed during seed development. This is a major seed protein components and form 40-50% of total protein content (Murphy et al., 1989).

Transcript level of the gene was measured at 2, 6 and 10 days of growth (Figure 21). *12S* shows no induction in the transgenic lines or wild type plants. However, the gene is stronger expressed in the transgenic lines at day 2. Later, the higher transcript levels could be only detected in TAK plants, even at day 10, however there the expression level is already decreasing. These results correlate well with the observed developmental alterations.

Since on the 10^{th} day of development the plants overcome the influence of anti-Bl scFv both at morphological and molecular level, these data suggest an important role for BRs in the seed development and during germination.

One of the LeB transgenic lines (TAK16152) overexpresses the *12S* gene at a much higher level as the other lines. The strong expression level was maintained constantly in the investigated time period. This observation can serve as a good starting point for further experiments in order to clarify the details of the effects of BRs on storage proteins.

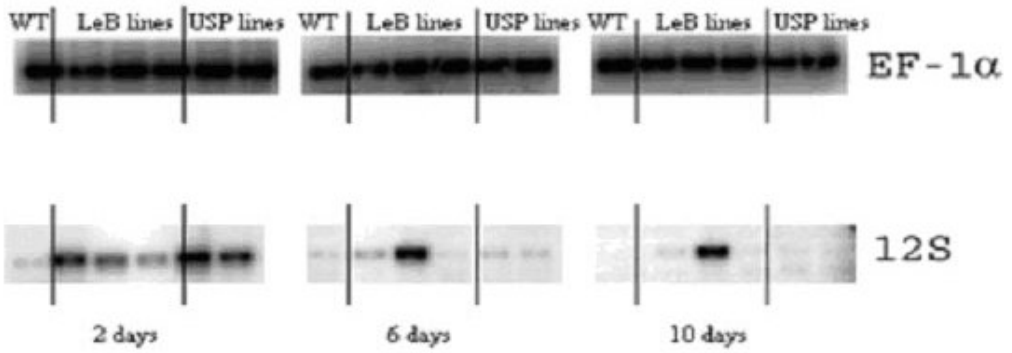

Figure 21. Expression of *12S* globulin in different time points of plant development.

Tissue samples were collected after 2, 6, and 8 days of growth in MS medium. **WT** – wild type plants; ER expression of anti-Bl scFv A16 under LeB4 promoter – **TAK1673, TAK19152, TAK1961** transgenic plants; ER expression of anti-Bl scFv under USP promoter – **TBU1648** and **TBU16104** transgenic plants. **EF1-α** - elongation factor-1alfa; **12S** - putative cruciferin 12S seed storage protein identical to 12S seed storage protein, gi808937

3.11. Seed protein content during germination.

During germination the seedling relies on stored materials for continued growth and development. One of these essential materials is reduced nitrogen, which is accumulated predominantly in form of seed storage proteins. *Arabidopsis* seeds contain two predominant classes of seed storage protein: legumin-type globulins (12S globulin or cruciferin) (Sjodahl et al., 1991), and napin-type albumins (2S albumins) (Krebbers et al., 1988). Procruciferin globulins are cleaved into two functional polypeptides, the α- and β-chain (Müntz, 1998) during seed maturation.

The cruciferin content of mature and germinating seeds was examined using Western blotting with polyclonal anti-cruciferin antibodies. As a control seeds of wild type plants were used. The protein extractions were done from single seeds. Equal amounts from each seed extract were loaded on PAGE-SDS gel. The signals were generated by chemiluminescence using ECL. The Western blot analysis indicated, that anti-Bl scFv A16 production did not result in major alterations in the accumulation of cruciferin (mature seed samples, Figure 22)

Compared with the wild type, distinct alterations in the protein profile were detected in transgenic lines after 36 hours of seed germination. Here, the α-chain of cruciferin with an approximate molecular mass 34 kD, remained longer intact compared to the wild type, where it degraded fast. This observation suggests a presumable role for BRs in releasing the stored proteins from the seed.

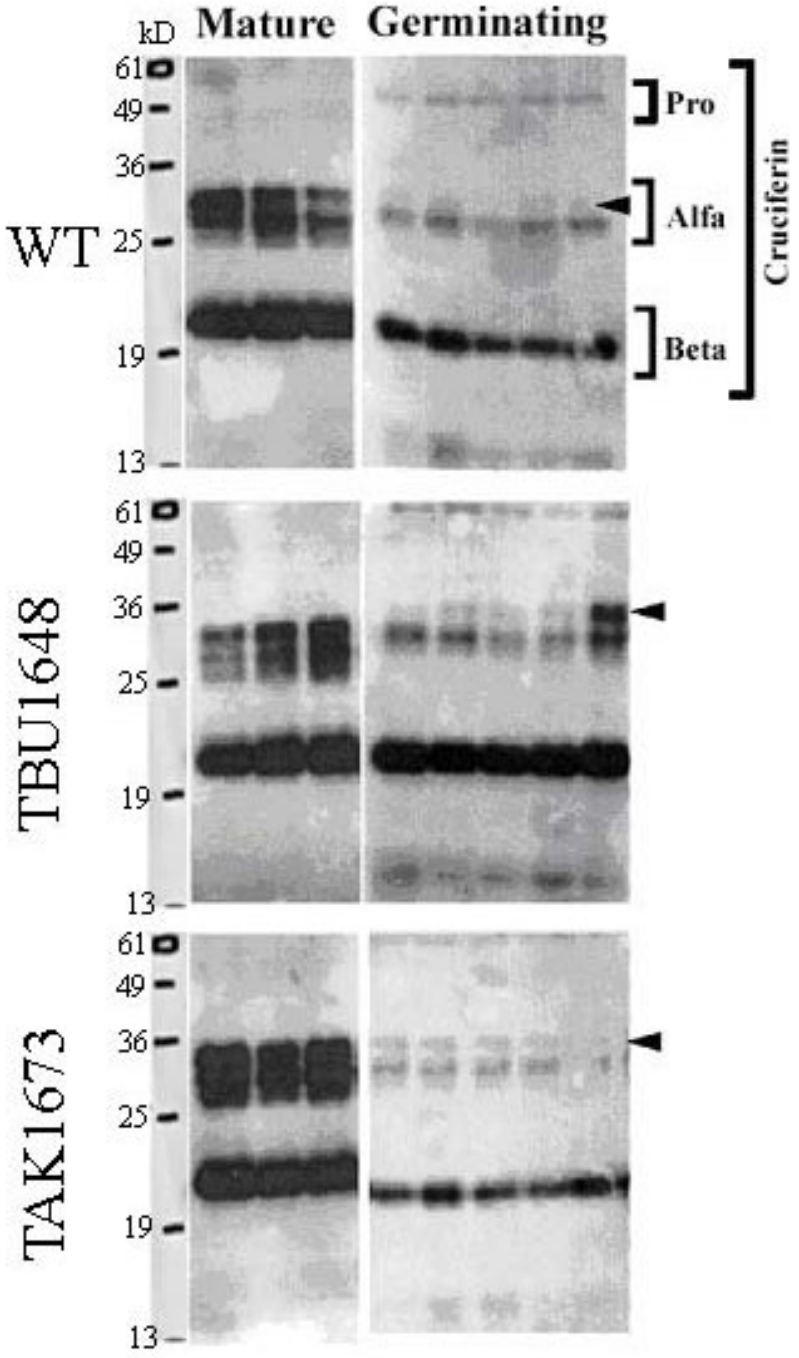

Figure 22. Western blot detection of crucifirin content in transgenic and wild type *Arabidopsis* mature seeds or germinating (36 h). **WT** – wild type plants; **TBU1648** – transgenic line with anti-Bl scFv A16 expression in ER under USP promoter; **TAK1673** – transgenic line with anti-Bl scFv A16expression in ER under LeB4 promoter. Arrow-heads indicate observed differences.

4. DISCUSSION

The present study was conducted to shed more light on brassinosteroids functions in the seeds and to lay the basis for further in-depth analysis for the identification of genes that exhibit transcriptional regulation by BRs. They are integrated in the complex signalling network and numerous BR effects appear to be mediated via a modulation of levels and sensitivities of other phytohormones. Correspondingly, BR-deficient and –insensitive mutants among another phenotypical alterations display delayed flowering and reduced fertility. In order to find out which genes are primarily affected by BRs in the seeds during seed maturation and germination, and also the developmental dynamics of the hormone action, the most suitable method is the immunomodulation. This method offers the opportunity to target macromolecules with high specificity and sensitivity and has been successfully used for modulation of ABA (Artsaenko, 1996) and jasmonate functions (ten Hoopen, 2002).

The intracellular expression of recombinant scFv antibodies targeted in to the ER expressed under control of seed-specific promoters USP and LeB4 have been used to functionally inactivate BRs in the seeds of transgenic plants by binding of antibody fragments. These promoters differed in strength and temporal activity during the early stages of seed development in tobacco (Fiedler and Conrad, 1995; Fiedler et al., 1993) and *Arabidopsis* plants (Figure 15). In *Arabidopsis* plants the scFv production under USP promoter control was detected at the 5th DAP whereas scFv expressed under the LeB4 promoter only becomes visible on the 8th DAP. This difference between promoter activations can be useful in understanding the dynamic of BRs functions during seed development. For this purpose, gene constructs were designed to allow the expression of scFv antibodies, their transport into and retention in the ER and expression in the cytosol and their specific detection in plant tissues. The signal peptide ensures the ER targeting, KDEL provides retention and stability of the expressed scFv, and c-myc is attached to scFv for specific and easy detection.

The maximal expression level of anti-Bl antibodies in the cytosol reached 0,5% of total soluble protein. Localization in ER was essential to obtain stable accumulation of the anti-Bl scFv protein in the seeds. Significantly higher accumulation of the scFv proteins in transgenic *Arabidopsis* seeds was achieved by retention of anti-Bl antibodies in the ER in comparison to their expression in the cytosol. The C-terminal KDEL tetrapeptide sequence was included to the expression cassettes in order to increase the scFv expression level and to stabilize the scFv in plant cytosole (Schouten et al., 1996). However the KDEL tetrapeptide does not improve the cytosolic accumulation level of every scFv protein. This had been reported before by ten Hoopen (2002). In plants with constitutive expression of scFv under CaMV 35S promoter control the accumulation of anti-Bl scFv in the cytosol of the seeds was not detectable or amount of accumulated scFv was low. Expression of anti-Bl scFv A16 in all experiments was higher in comparison to production of anti-Bl scFv A30. The expression level of a particular scFv is obviously coding-sequence dependent. The plants with scFv A16 expression under USP and LeB4 promoter control were selected to obtain plants with high expression level of anti-Bl scFv in the seeds.

The seed protein extracts from the plants producing anti-Bl scFv antibodies were tested by ELISA. The recombinant anti-Bl scFv specifically bound to the 24-CS-BSA conjugate *in vivo*. The highest values were received from transgenic lines with anti-Bl scFv A16 expression under the control of LeB4 promoter. These results correlate with the western blot analysis, where highest scFv accumulation was

detected in the same lines with highest ELISA values. The capability of the anti-Bl scFv antibodies to bind 24-epiCs antigen confirmes their functionality *in vivo* and the detected accumulation of scFv by western blot analysis can be extrapolated on anti-Bl scFv antibodies activity.

Plants store proteins in embryonic and vegetative cells to provide carbon, nitrogen, and sulfur resources for subsequent growth and development. Storage proteins accumulate primarily in the protein storage vacuoles of terminally differentiated cells of the embryo and endosperm and as protein bodies directly assembled within the ER. PSV consists of the protein matrix, a proteinaceous crystalloid, phytin inclusions, and intravacuolar vesicles derived by autophagy (Herman and Larkins, 1999). The examination of ultrastructural alterations in the mature transgenic seeds with anti-Bl scFv antibodies expression have shown changes in content and shape of PSV. Wild type plants have protein bodies in the shape of sphere in contrast to transgenic lines. The phytin inside of PSV occurred much more often in the cells of transgenic plants (chapter 3.7).

The promoting effect of BRs on seed germination has been already shown by several researchers (Steber and McCount, 2001; Takeuchi et al., 1995; Ullah et al., 2002). Significant differences between transgenic and wild-type plants were observed during seed germination *in vitro* on MS medium. The transgenic plants, producing anti-Bl scFv antibodies exhibited a germination and growth delay. The differences could be observed from germination till the 10^{th} day of development. These results are consistent with the observation, that at this time-point the BS antibodies are not present any longer in the seedlings. After the plant is not dependent on the nutrient content of the seed and can synthesize and acquire the necessary compounds from the medium, transgenic plants overcome the germination delay, showing no more difference to the wild type plants. We propose that BRs have an activator effect on mobilizing the nutrient stores of the seed during germination. Thus, the lack of BRs in transgenic seedlings would result in an ineffective promoter signal of the release of nutrients, leading to a delayed premature development. Another evidence for this proposed function was obtained in an experiment where transgenic plants were transferred from *in vitro* to the soil. The growth delay could be prolonged in these plants by transferring them *in vivo* in contrast to wild type plants. The developmental delay could be maintained until 20 days of growth. The observed longer growth retardation is possibly due to the nutritional difference between the MS medium and soil. The transgenic plants recover faster on rich medium, which contains ready available nutrients, reflecting on the importance of BRs in mobilizing such compounds.

The specific ability of transgenic *Arabidopsis* plants, containing anti-Bl scFv antibodies in the ER, on Bl-mediated inhibition of seed germination confirms that the anti-Bl antibodies are active in the transgenic plants and can be used as a reliable tool for immunomodulation of BRs functions.

The promotive effect of BL on root growth in rice seedlings was shown by Konishi and Komatsu (2003). They have analyzed the composition of the protein extract of BL treated roots and found that the content of glutathione S-transferase (GST) homolog proteins was increased. It is known, that GSTs are abundant proteins encoded by a highly divergent gene family. Soluble GSTs form dimers, each subunit of which contains active sites that bind glutathione and hydrophobic ligands. Plant GSTs attach glutathione to electrophilic xenobiotics, which tags them for vacuolar sequestration. The role of GSTs in metabolism is unclear. Their complex regulation by environmental stimuli implies that they have important protective functions (Edwards et al., 2000). GSTs also catalyse glutathione-dependent isomerizations and

the reduction of toxic organic hydroperoxides. GSTs might also have non-catalytic roles as carriers for phytochemicals. Additionally GSTs have high affinity to auxins and cytokinin. Their contribution in hormone homeostasis has been suggested (Gonneau et al., 1998; Marrs, 1996). Root length measurements of transgenic plants with anti-Bl scFv antibodies expression have shown significant differences. The roots of transgenic lines were 30-40% shorter. The expression level of two genes with homology to glutathione trasferase decreased in transgenic plants. As already suggested by Konishi and Komatsu (2003), BRs can play a role in GSTs regulation in roots.

Very recently several reports have been published where BR-regulated genes were identified compared BR-treated plants with control plants (Zurek et al., 1994; Hu et al., 2000; Müssig et al., 2000) and in comparison of BR-deficient plants *versus* wild-type plants (Müssig et al., 2002). Gene expression profiles of transgenic lines with immunomodulated BRs, received with Affymetrix Arabidopsis Genom Arrays technique gave information about changes in transcript levels during seed maturation and germination. The chip contains more than 22,500 probe sets representing approximately 24,000 genes. RNA samples were prepared from the 6^{th} day old seedlings grown *in vitro* on MS medium. Data obtained by hybridization were analyzed by Microarray Suite software. The Affymetrix Microarray Suite program is performing several tests: single array analysis and comparison analysis (experiment versus baseline). The first test is providing initial data for comparison analysis. This test indicates the presence or absence of the signal on the GeneChip. In the second test two GeneChip probe arrays of the same type are compared against each other in order to detect and quantify changes in gene expression. In our experiments hundreds of genes show an altered expression level in transgenic plants *vs.* wild type. We found, that the ratio of differentially expressed genes *versus* wild type was 37%. In order to select those genes where the changes were significant we applied the most stringent requirements: (a) the calculated values must be labelled like INCREASE or DECREASED; (b) the signal have to be present in one of the baseline or experimental samples, if the signal is close to zero in both samples then the change of the signal is considered to be not significant; (c) the signal log ratio must be more than one (fold change 2 times). After such selection approximately 35% from these genes were found to be induced in transgenic plants in comparison to wild type. Genes were sorted according to their functions on several groups: seed storage proteins; heat shock proteins; LEA proteins; ribosomal proteins; genes involved in DNA transcription and translation, transcriptional factors; proteases; kinases; enzymes; and others. Genes with unknown functions are not presented in the following.

Some of the genes with altered expression level in transgenic plants belong to gene families members of which were shown to interact with brassinosteroids. Other genes, for example those encoding seed storage proteins, were not yet brought in context with BRs The possible connections to BR response of the genes with the altered expression in transgenic plants are discussed here.

The expression of several genes of seed storage proteins was induced in transgenic plants with anti-Bl scFv antibodies expression. The seeds of plants often contain large amounts of proteins, which are subjected to extensive proteolytic processing during seed development and subsequent germination. The major classes of seed storage proteins are legumin-type (12S) and napin type (2S). The expression levels of the genes encoding the representatives of both classes (five genes of 2s albumins and tree genes of legumin proteins) was found to be increased in transgenic lines. Additionally the expression of other members of the legume seed proteins, the

Bowman-Birk-type trypsin inhibitors was also expressed in transgenic plants at a high level. Sequence studies of the trypsin inhibitors from a number of legume species suggest that many of the inhibitors undergo a limited shortening at the amino terminus during seed development. However, during germination, the inhibitors appear to function as storage proteins (Wilson, 1988.). From another side the expression level of the genes encoding proteases decreased. According to these data we propose a model of BRs nutrient mobilization effect through induction of proteases during seed germination. The increased level of the production of seed storage proteins can be an answer to inadequate protein degradation resulting in a deficiency of released nutrients, which are initially required for the germination and development. The western blot analysis of cruciferin content in seeds during germination has shown the immobilization delay of cruciferin content in transgenic seeds. This can be a consequence of low protease activities. Another possibility is, that the longer presence of cruciferin α–chain in germinating seeds of transgenics, is a consequence of the prolonged cruciferin synthesis observed in the transgenic plants up to six days after germination.

The monitoring of correlation between storage proteins and sHSPs accumulation of both types of protein in tobacco seeds during seed maturation and germination has shown that the expression of sHSPs and globulins is activated simultaneously at about the 17th day after anthesis (Lubaretz and zur Nieden, 2002). During germination the seeds sHSPs and storage proteins content decreases parallel. Furthermore, in embryos of transgenic tobacco plants, which do not contain any protein bodies or storage proteins, no sHSPs were found. Thus, the occurrence of sHSPs in perennial plant storage organs seems to be associated with the presence of storage proteins (Lubaretz and zur Nieden, 2002). According to the Affymetrix data, HPS and seed storage protein genes are expressed differently in transgenic plants with anti-Bl scFv antibody expression. The transcription of storage proteins is induced during germination, but transcription of HSP is repressed. As previously shown, treatment of *Brassica napus* seedlings with EBL leads to a significant increase of their basic thermo tolerance and results in higher accumulation of some HSP as compared to untreated seedlings (Dhaubhadel et al., 2002). The authors report, that increased accumulation of HSPs in EBL treated seedlings results from higher HSPs synthesis, even if the mRNA levels are lower then in untreated seedlings, and that several translation and elongation factors are present at significantly higher levels in EBR-treated seedlings. In addition to protective function, HSPs can also regulate translation (Brodsky et al., 1998; Ling et al., 2000). We propose that the translation during seed germination can be regulated by BRs with HSP mediation.

Several studies have speculated that sHSPs may function to protect cellular components during seed desiccation and/or during rehydration (Coca et al., 1994; DeRocher and Vierling, 1994; Alamillo et al., 1995; Wehmeyer et al., 1996). They are not sufficient, but could be necessary for dormancy. At the end of embryogenesis, seeds desiccate and become dormant, so that the embryo can survive for a longer time. Desiccation tolerance allows the seed to be dried and stored for an extended period (Ingram and Bartels, 1996). The molecular and biochemical events that govern these processes are incompletely defined, although LEA (late embryogenic abundant) proteins have been hypothesized to be involved (Finkelstein, 1993; Ingram and Bartels, 1996; Kermode, 1997). mRNAs of some transcripts of LEA proteins are accumulated during this late seed maturation phase and the process of accumulation is regulated by ABA. The LEA class comprises genes that code for LEA proteins that are thought to be involved in desiccation tolerance (Hoekstra et al.,

2001). In *abi3* mutant seeds, the expression of multiple genes from LEA class is reduced compared with that in wild-type seeds (Parcy et al., 1994; Nambara et al., 1995, 2000). The same difference was observed for the expression of sHSPs genes. As shown by Wehmeyer and Vierling, (2000), desiccation-intolerant mutants *abi3-6*, *fus3-3*, *lec1-2*, and *line24*, had significantly reduced (1%–2% of wild type) or undetectable HSP´contents. The expression level of LEA and HSP genes is decreased in transgenic plants with anti-Bl antibodies expression. These data indicates the role of BRs in forming desiccation tolerance in the seeds.

Plant cells often use receptors at the cell surface to sense environmental changes, and then transduce this information via activated signaling pathways to trigger adaptive responses. The receptor-like protein kinases (RLKs) are transmembrane proteins with cytoplasmic serin/theonin kinase domains and divergent extracellular domain. RLKs can potentially bind an array of molecules, including carbohydrates, polypeptides, microbial cell-wall components and steroids, depending on their extracellular domain (Shiu and Bleecker, 2001). In *Arabidopsis*, the RLK gene family contains more than 600 member, many of those are likely to respond to the external challenges presented by an ever-changing environment. RLKs are involved in hormonal response pathways, cell differentiation, plant growth and development, self-incompatibility, symbiont and pathogen recognition (Morris and Walker, 2003). According to Affymetrix results, several RLKs have altered expression level in transgenic plants with immunomodulated BRs functions. We propose that BRs act through RLKs regulation. These results well correlated with the previous data obtained showing very wide range of BRs physiological action (chapter 1.4).

In several eukaryotic organisms the mRNA expression for ribosomal proteins (RPs) is tightly regulated at the translational level. Synthesis of RPs was also observed under conditions of transcription inhibition, suggesting the presence of stored-RP transcripts in the embryonic axes (Beltran-Pena et al., 1995). The initial protein synthesis during seed germination is supported by stored mRNAs. The characterization of some stored mRNA identified ribosomal proteins (RPs) within the population of stored mRNAs. The newly synthesized RPs are already assembled in the ribosome as early as 3h of germination. Within this we suggest that studying BRs functions in regulation of RP synthesis and mRNA storage needs analysis of early stages of seed germination. (Beltran-Pena et *al*., 1995). BR-regulated transcription factors may provide important insights into BR actions.

Arabidopsis thaliana homeobox gene (ATHB) -4 gene product belongs to the homeodomain-leucine zipper (HD-ZIP) II family which are characterized by the presence of both a homeodomain and a leucine zipper motif (Sakakibara K. et al., 2001). ATHB-4 gene shares 47% homology with ATHB-2 (Carabelli et *al*., 1993), also known as HAT4, (Schena et al., 1993). The ATHB-2 and -4 genes have peculiar regulation by light quality. (Carabelli et *al*., 1993, 1996). The ATHB-2 gene is expressed during the vegetative and reproductive phases of plant growth. A significant increment in the amount of ATHB-2 transcripts is observed in flowering plants. Remarkably, far-red(FR)-rich light treatment of Arabidopsis plants results in a rapid and strong induction of the ATHB-2 expression. This light treatment also induces the accumulation of ATHB-4 transcripts. The transgenic plants with altered ATHB-2 expression exhibit a series of developmental phenotypes (Schena et al., 1993). A direct involvement of these members of the HD-ZIP II family in light-mediated growth phenomena was suggested. It has been already demonstrated that BRs plays a role in light-regulated development and gene expression in Arabidopsis

(Chory et al., 1996; Szekeres et al., 1996; Li et al., 1996; Nagata et al., 2000). We suppose that the HD-ZIP II family belongs to the BRs light-response signaling.

Expression studies have shown that the light regulation of the *ATHB-2* gene is quite complex, involving at least three distinct phytochromes. In etiolated seedlings, the gene is expressed at relatively high levels. In young seedlings and mature plants, *ATHB-2* is expressed at low levels under R-rich light, but is rapidly and strongly induced by FR-rich light. Return the plants to red(R)-rich light results in an equally rapid decrease of the *ATHB-2* mRNA levels. Kinetics of FR-rich light induction and its reversibility by R-rich light performed in *phyB* and *phyA phyB* plants revealed that the *ATHB-2* gene is reversibly regulated by changes in the R:FR ratio largely through the action of a phytochrome other than A or B and secondarily by phytochrome B (Carabelli et al., 1996 Steindler et al., 1997). Expression of two genes encoding cytochrome P450 was decreased in transgenic plants. This protein is known to catalyze C-2 hydroxylation in brassinosteroid biosynthesis and P450 is dark-induced and predominantly expressed in the rapidly elongating zone of etiolated pea epicotyls. Overexpression of the cytochrome P450 results in enhanced hypocotyl growth even in the light. A dark-induced small G protein, pea Pra2, regulates a variant cytochrome P450. Transgenic plants with reduced Pra2 exhibit a dark-specific dwarfism, which is completely rescued by exogenous brassinolide and proposed that Pra2 are molecular mediators for the cross-talk between light and brassinosteroids in the etiolation process in plants. (Kang et al., 2001). We suppose that in *Arabidopsis* plants BRs are regulating expression of P450 by a distinct way, not via Pra2.

We also would like to pay attention on increase in expression of CLE7 (CLAVITA7) gene. It is known that CLE genes are down regulating the expression of WUS (WUSCHEL) gene, which promotes the formation and maintenance of stem cells (Brand et al., 2002). The ability of meristem to continuously produce new organs depends on the activity of their stem cell population. The ability of BRs to promote vegetative growth in plants had been already described in details (see chapter 1.4). We speculate that BRs can cause a negative regulation in the pathway, which restricts stem cell number by inhibition of CLE genes expression and with it promotes plant growth.

The expression monitoring experiments of the genes with the altered expression profile in transgenic plants with BR starvation in seeds have identified several new BR-regulated genes. New functions of BRs in seed formation and germination are proposed. These results built a good basis for further investigations to gain a deeper insight into the regulation of gene expression by BRs in seed development.

5. ABSTRACT

Brassinosteroids represent a new class of plant hormones essential for growth and development. Mutant analysis revealed the biosynthesis of these steroids, as well as their involvement in signal transduction and plant growth. Since BRs are involved generally in plant development, a function in seed germination was presumed. In the present work we wanted to investigate, how contribute brassinosteroids to seed ripening and what function they have in germination. To achieve this general goal, the function of brassinosteroids will be blocked by immunomodulation in seeds. This should enable us to study the role of brassinosteroids in seed maturation and germination and to find candidate genes possibly regulated by brassinosteroids during germination. Since Brassinosteroid mutants do not make seeds, immunomodulation is the most suitable method for the silencing of the hormone function in seeds.

As an important tool recombinant single-chain Fv antibodies were selected, that either bind to brassinolide, castasterone and there isomer forms 24-epicastaserone and 24-epibrassinolide (A30), or to 24-epicastaserone and 24-epibrassinolide (A16). Their coding sequences were cloned into plant expression vectors for production of these antibodies in the endoplasmatic reticulum of seeds. The A30 and A16 antibody coding genes were used to construct expression cassettes for ER retention under the control of seed specific USP and LeB4 promoters.

The generated transgenic *Arabidopsis thaliana* and *Nicotiana tabacum* plants stably and heritably accumulated the anti-brassinosteroids recombinant antibodies in the endoplasmic reticulum of seed cells. Non-segregating homozygous transgenic lines were selected by the classical Mendel analysis. The plants accumulated scFv A16 antibodies at a higher level then scFv A30. The recombinant antibodies from plant were verified *in vitro* for specific binding to 24-epicastasterone, essential for the physiological activity *in vivo*.

The functions of brassinosteroids were investigated by immunomodulation at different time points of seed development. For this propose, the recombinant antibodies were either expressed under control of the USP promoter, were transgenic scFv proteins could be detected already at day 5 after pollination, or under control of the Legumine B4 promoter, were scFv expression was measured firstly at day 8 after pollination. The transgenic lines performed several morphological and physiological

alterations. Mature transgenic *Arabidopsis* seeds carried alterations in number and shape of phytin granules and protein bodies, reflecting to inadequate nutrient storage. Transgenic plants exhibited delay in germination and development after germination. The developmental delay can be prolonged by transferring transgenic plant from *in vitro* culture to soil. The modulation of brassinosteroid function in the germinating seeds triggered modifications at a molecular level as well. Inadequate expression of several genes was detected. Genes belonging to the families of seed storage proteins, heat shock proteins, kinases and some others were differentially expressed in the transgenic plants, presuming a function in the seed germination and nutrient release for the BRs. The alterations of the transgenic lines in the nutrient status could be demonstrated by studies on the expression level of the cruciferin gene (described seed storage protein) both at transcript and protein level.

6. Zusammenfassung

Brassinosteroide, eine neue Klasse von Phytohormonen, haben essentielle Funktionen bei Wachstums- und Entwicklungsprozessen. Durch Analyse entsprechender Mutanten wurde sowohl die Biosynthese dieser Steroide als auch ihre Rolle bei Signaltransduktionsprozessen und beim Pflanzenwachstum untersucht. Da Brassinosteroide generell an der Pflanzenentwicklung beteiligt sind, wird eine Funktion bei der Samenentwicklung angenommen. In der vorliegenden Arbeit sollte untersucht werden, in welcher Weise Brassinosteroide zur Samenentwicklung beitragen und welche Funktion sie bei der Keimung haben. Um dieses generelle Ziel zu erreichen, wurden die Brassinosteroidfunktionen im Samen durch Immunmodulation blockiert. Auf diese Weise sollten die Einflüsse von Brassinosteroiden auf Samenreifung und Keimung untersucht werden. Gene, die während der Keimung möglicherweise durch Brassinosteroide reguliert werden, sollten identifiziert werden. Da Brassinosteroidmutanten keine Samen produzieren, stellt Immunmodulation die geeignete Methode dar, um solche Hormonwirkungen im Samen spezifisch zu unterbinden.

Als wichtiges Werkzeug wurden spezifische rekombinante Einkettenantikörper selektiert, die entweder an Brassinolid, Castasteron und die Isomere 24-Epibrassinolid und 24-Epicastasteron (Antikörper A30) oder nur an 24-Epiobrassinolid und 24-Epicastasteron (Antikörper A16) spezifisch binden. Die entsprechenden kodierenden Sequenzen wurden in Pflanzenexpressionsvektoren kloniert , die eine Lokalisierung dieser Antikörper im Endoplasmatischen Retikulum von Samenzellen gewährleisten. Hierfür wurden für beide Einkettenantikörpergene die samenspezifischen Promotorem USP und Legumin B4 benutzt.

Transgene *Arabidopsis thaliana*- und *Nicotiana tabacum*-Pflanzen akkumulierten die rekombinanten anti Brassinosteroidantikörper stabil und vererbbar im Endoplasmatischen Retikulum von Samenzellen. Nichtsegregierende homozygote transgene Linien wurden durch klassische genetische Analye selektiert. Diese Pflanzen akkumulierten den Einkettenantikörper A16 stärker als den Einkettenantikörper A30 im ER. Die rekombinanten Antikörper aus den Pflanzen banden *in vitro* spezifisch an 24-Epicastasteron. Diese Bindungsaktivität ist für die physiologische Wirkung *in vivo* essentiell. Die Brassinosteroidfunktionen wurden zu verschiedenen Zeitpunkten der Samenentwicklung durch Immunmodulation

untersucht. Die rekombinanten Antikörper wurden zu diesem Zweck entweder unter Kontrolle des USP-Promotors oder unter Kontrolle des LeguminB4-Promotors exprimiert. Bei Expression unter Kontrolle des USP-Promotors konnten die transgenen Proteine schon ab Tag 4 nach der Bestäubung, in großen Mengen ab Tag 8 nach der Bestäubung, nachgewiesen werden. Bei Expression unter Kontrolle des LeguminB4-Promotors konnten die rekombinanten Antikörper ab 8, in großen Mengen ab Tag 9 nach der Bestäubun nachgewiesen werden.

Die transgenen Pflanzenlinien wiesen verschiedene morphologische und physiologische Veränderungen auf. Reife transgene *Arabidosis*-Samen zeigten Veränderungen in Menge und Form der Phytin-haltigen Proteinkörper, was als Folge inadäquater Nährstoffeinlagerung zu deuten ist. Die transgenen Pflanzen zeigten verzögerte Keimung und verzögerte Entwicklung nach der Keimung. Diese Entwicklungsverzögerung kann durch Transfer der Pflanze aus der *in vitro*-Kultur in Erdkultur verlängert werden. Die Modulation der Brassinosteroidfunktionen in keimenden Samen verursachte auch Veränderungen auf molekularer Ebene. Inadäquate Expression wurde für verschiedene Gene nachgewiesen. Gene aus den Familien der Reserveproteine, Hitzschockproteine, Kinasen sowie einige andere wurden differentiell in den transgen Pflanzen expriniert, was auf eine Funktion der Brassinosteroide bei der Keimung und bei der Nährstoffmobilisierung schließen läßt. Veränderungen der transgenen Linien im Nährstoffstatus wurden durch Studien der Expression des Cruciferringenes sowohl auf Transcript- als auch auf Proteinebene demonstriert.

7. References

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Declaration

I hereby declare that the work presented in this manuscript is my own and was carried out entirely with help of literature and aid cited in the manuscript.

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