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DIPLOMARBEIT

Titel der Diplomarbeit

HEAT SHOCK ENHANCER ELEMENT-MEDIATED EXPRESSION OF ß-GLUCURONIDASE IN *ARABIDOPSIS THALIANA*

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1. Introduction

1.1. Principles of Inducible Genetic Systems

Inducible genetic systems have a great potential in both molecular genetics to study biological functions of induced proteins in almost any field of biology and in biotechnology, where controlled expression is essential for the production of recombinant proteins. Therefore the requirements for a good inducible system are high: 1.) Without induction the expression of the controlled gene should be the least leaky possible. 2.) Induction should be dose-dependent and induction levels should span over at least three orders of magnitude. 3.) The expression pattern should be homogenous in all tissue of the organism. 4.) Inducing conditions should bare minimal consequences on the organism's physiology, fitness and viability (Gatz, 1997). In plants, prevalent conditional gene-expression systems use chemically regulated heterologous promoters (Padidam, 2003) with chemical inducers such as tetracycline (Gatz, 1997), steroids (Aoyama and Chua, 1997; Zuo et al., 2000; Zuo et al., 2002) and ethanol (Caddick et al., 1998; Roslan et al., 2001). Induction in such systems reaches up to a few hundred folds but has several drawbacks such as promotor leakiness, non-uniform uptake and toxic effects of the inducing chemicals (Padidam, 2003).

In all cells of organisms gene expression is divided into constitutively active expression and conditional expression. Constitutive gene expression is reserved for genes that are required during the complete life time of the cell, and are also called house keeping genes. They are necessary for fundamental cell functions, ensuring its survival. Conditional gene expression serves on the one hand as a response mechanism to changing environmental conditions, on the other hand as a regulatory mechanism during the development and growth of multicellular organisms. In most cases the inducing factor (be it a chemical compound or environmental cues) interacts with an endogenous responsive transcription factor, which is then (and only than) able to assist in the recruitment of RNA-Polymerases to initiate transcription of the target gene. This target gene is usually under control of a minimal promoter, so it is genetically silent in the absence of the inducing factor.

1.2. Inducible Gene Expression Systems in Plants

The use of inducible systems for various transgenes has become a very important tool in the elucidation of plant gene function. It allows a more precise temporal and spatial control over the ectopic expression or misexpression of the specific transgene. In contrast to transactivation systems, where a latent transgene under the control of a minimal promoter is activated by a transcription factor encoded on a second vector, inducible systems usually rely on the use of a single vector. This vector contains a transgene, often also a reporter gene to monitor the induction levels, both under control of a minimal promoter, and a responsive element for specific chemicals or environmental conditions such as heat.

1.2.1. Chemically inducible systems

Since the initial work of Christiane Gatz, Robin Wilde and their colleagues in the early 1990s, a large number of chemically inducible systems have been described (Table 1).

Chemically Inducible System	Inducer	References
De-repressible TetR	Tetracycline	Gatz et al. (1992)
Inactivable tTA	Tetracycline	Weinmann et al. (1994)
Dual control TGV	Dexamethasone, Tetracycline	Böhner and Gatz (2001), Böhner et al. (1999)
Copper-inducible Ace1	Copper	Mett et al. (1993)
Dexamethasone-inducible GVG, LhGR	Dexamethasone,	Aoyama and Chua (1997), Craft et al.(2005), Samalova et al. (2005)
Insecticide-inducible GVGE, GVE, VGE	Tebufenozide, methoxyfenozide	Koo et al. (2004), Martinez et al. (1999), Padidam et al. (2003)
Oestrogen-inducible ER-C1, XVE	17-ß-oestradiol	Bruce et al. (2000), Zuo et al. (2000)

Ethanol-inducible AlcR	Ethanol/ acetaldehyde	Caddick et al. (1998), Roslan et al. (2001), Salter et al. (1998)

Table 1 Chemically inducible systems for plants (Moore et al., 2006)

The most important of these systems are described below.

1.2.2. The alc System: chemical induction by ethanol

Ethanol-inducible expression from the AlcA promoter in Arabidopis

The system now in discussion uses a natural rather than an artificial transcription factor, which originates from *Aspergillus nidulans* (Caddick et al., 1998; Roslan et al., 2001; Salter et al., 1998). The *A. nidulans* ALCR transcription factor responds primarily to acetaldehyde, a product of ethanol metabolism. To obtain an ethanol inducible system, the gene of interest is cloned under the control of the *palc*A promoter, which consists of the upstream promoter sequences of the *A. nidulans* *alc*A gene fused to a minimal CaMV 35S promoter (-31 to +5). In the absence of ALCR, *palc*A is silent in Arabidopsis even under conditions that generate endogenous ethanol (Roslan et al., 2001), so endogenous transcription factors do not recognize the *alc*A-derived sequences.

Figure 1. In the presence of ethanol or acetaldehyde, the Aspergillus nidulans ALCR transcription factor (alcR) drives expression from the palcA promoter by binding to upstream sequences (alcA) from the A. nidulans alcA locus.

The alc system has been used in several studies in Arabidopsis for overexpression of foreign or endogenous proteins, for tissue-specific inducible expression (Deveaux et al., 2003), for conditional complementation of a mutant phenotype (Laufs et al., 2003; Maizel and Weigel, 2004) and for expression of RNAi (Chen et al., 2003; Ketelaar et al., 2004; Lo et al., 2005). Several methods of induction have been successfully used and the characteristics of the system in Arabidopsis have been described carefully by Roslan et al. (2001).

Induced GUS levels are low (25-40 pmol min^{-1} μg^{-1}) compared to other systems. When fully induced (2% v/v soil drench) the *alc* switch exhibits a 10^3-fold induction from undetectable levels but in agar-grown seedlings the induction factor is less than 50-fold owing to a substantial increase in uninduced expression (Deveaux et al., 2003; Roslan et al., 2001). As the *palc*A promoter is not active in wild-type plants, this increased activity almost certainly results from the accumulation of endogenous metabolites that activate ALCR. Anoxia and tissue culture conditions are thought to promote the accumulation of these metabolites precluding the use of this system in tissue culture (Roberts et al., 2005). For this reason, the alc system is rarely used to regulate the activity of genes that affect physiology or development at very low expression levels. In one case, Howles et al., 2005 reported the regulated expression of a protein that causes plant death upon induction. Leaky expression in vitro may not compromise many overexpression or RNAi experiments that depend on strong induction to generate phenotypes. Conditional complementation is also possible as shown by LEAFY>alcR>LEAFY in a leafy background in which the leafy phenotype was lost only sporadically in uninduced individuals of each transgenic line (Maizel and Weigel, 2004). Deveaux et al. (2003) note that auto-induction of ALCR in culture medium can be minimized if roots are prevented from penetrating the medium, for example by increasing the agar concentration or inclining the plates. Another, more secure way is to introduce *palc*A constructs initially into wild-type plants then, once transformants have been selected in vitro, to cross to promoter::alcR lines and perform subsequent studies in soil. For application of ethanol it is common to add it as a drench to soil (5 ml per 100 ml soil) or agar plate (1ml per 12 x 12 x 1 cm plate)(Roslan et al., 2001). Alternatively it can also be applied as a vapour if a tube containing 50-100% ethanol is placed in a Petri dish with seedlings or in a plastic bag or closed propagator containing soil-grown plants (Deveaux et al., 2003; Roslan et al., 2001). Enclosure increases the efficiency of the induction and prevents ethanol vapour from inducing expression in neighbouring plants. The volatility of ethanol means that it is readily applied to whole plants, be they seedlings in a Petri dish or mature plants in the greenhouse, and ethanol can penetrate to internal meristematic cells (Deveaux et al., 2003; Roslan et al., 2001). The rapid uptake and metabolism of ethanol means that the induction period can be limited. Expression begins within 1 or 2 h of treatment, with transcripts reaching their maximum abundance within 6–8 h and disappearing within 18–48 h after removal of the inducer (Deveaux et al., 2003;

Laufs et al., 2003; Roslan et al., 2001). Localized induction on the other hand is difficult to achieve because of the mentioned volatility. Soil drenches need to be repeated at 0,5- to 3-day intervals to sustain induced expression and induction of the luciferase protein, which is unstable, was not successful (Roslan et al., 2001). Due to the toxicity of ethanol, Roslan et al. (2001) caution that the induction conditions must be optimized for each experiment for the optimal compromise between induced expression and adverse effects on plants. The concentration where ethanol shows inhibitory effects is influenced by the conditions under which the plants are grown. Induction by ethanol vapours is advised for short episodes of induction as it maximizes the efficiency of induction yet minimizes the toxicity of ethanol (Roslan et al., 2001). However, the requirement to keep plants enclosed can affect their biology if treatment is extended beyond a day or two (Roslan et al., 2001). Silencing of the *alc* system was observed in some selected lines by Roslan et al- (2001), though it was correlated with loss of ALCR expression from the CaMV promoter suggesting that palcA remains accessible to activation over many generations (Roslan et al., 2001).

Ethanol-inducible expression from the AlcA promoter in other species

The *alc* system was first described in tobacco where it was used for expression of the CAT reporter gene and a cytosolic invertase (Caddick et al., 1998; Salter et al., 1998). Since then it has been used in few studies of gene function in this species (Chen et al., 2003; Lo et al., 2005; Schaarschmidt et al., 2004). Similar considerations apply to the use of the *alc* system in tobacco and Arabidopsis with respect to activation by endogenous ethanol and optimization of induction regimes (Salter et al., 1998; Sweetman et al., 2002). Vapour induction is recommended over soil watering and the induced GUS activities compare favourably with CaMV::35S GUS values for tobacco (Sweetman et al., 2002). Schaarschmidt et al. (2004) show that acetaldehyde is a more effective inducer than ethanol in roots where it has the advantage that it is not transported from the site of application. However, with increasing acetaldehyde concentrations, induced expression levels in roots peak sharply at 0.1% raising concerns that the compound is toxic at or below fully inducing levels. Repeated treatment with suboptimal concentrations was more effective at inducing GUS activity and was not observed to alter plant growth, though

glyceraldehydes phosphate dehydrogenase (GAPDH) activities were reduced transiently by 40%. Induced GUS activity remains high for 2 to 4 days after application of acetaldehyde, then decreases but does not return to uninduced levels for at least 14 days. Induction with acetaldehyde in leaves does not work well but Chen et al. (2003) found that RNAi could be confined to a single leaf bagged with ethanol vapour. Endogenous genes in tobacco can trigger ALCR, so genes that interfere with regeneration must be introduced into an ALCR-free background. However, this precludes its use in cultures such as the BY-2 suspension culture. To overcome this, Roberts et al. (2005) fused a rat GR ligand-binding domain to ALCR to make a dexamethasone-inducible factor that targets *palcA*.

Furthermore the *alc* system has been used in potato, tomato and oil-seed rape (Garoosi et al., 2005; Junker et al., 2003, 2004; Sweetman et al., 2002). In these species, the system has similar limitations and characteristics to those described for tobacco with respect to auto-induction in vitro (Roberts et al., 2005), but in potato there is the added drawback that mature soil-grown tubers exhibit very high activation of ALCR from endogenous inducers (Junker et al., 2003). The tuber is clearly an important focus for research in potato but is far from ideal for the rapid and homogeneous application of inducers. In young detached tubers, ethanol vapour is able to penetrate to the centre but it takes 7 days to achieve homogeneous induction (Sweetman et al., 2002). Maximum induction is achieved with 1% ethanol but 5% is substantially worse (Junker et al., 2003) raising concerns that full induction may occur close to or above toxic levels. Vreugdenhil et al. (2006) reported that ethanol affects important aspects of tuber physiology at inducing concentrations.

In tomato hydroponically grown seedlings as well as soil-grown mature plants have been successfully induced with 0,1% or 10% respectively, with similar toxic side effects at concentrations close to maximum induction (Garoosi et al., 2005). Foliar application, which is effective in other species, was ineffective in tomato, which were sometimes killed after being sprayed with 7% ethanol (Garoosi et al., 2005).

1.2.3. Hormone dependent inducible systems

GVG and VGE in Arabidopsis

The first system based on steroid-hormone receptors was the GVG system comprising residues 1-74 of Gal4 (DNA binding domain), the *Herpes simplex* derived transactivation domain VP16 and the ligand-binding domain of a rat glucocorticoid receptor. The MCS (Multiple cloning site) is preceded by a promoter containing six copies of the Gal4 UAS and a minimal CaMV 35S promoter (Aoyama and Chua, 1997).

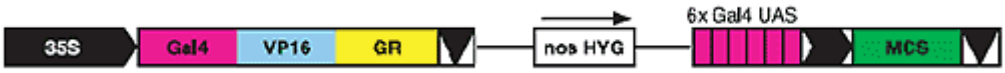

Figure 2. GVG steroid-hormone inducible system, pTA7001 (right border on left, left border on right). Single T-DNA vector for dexamethasone-inducible expression. The chimeric transcription factor GVG comprises residues 1–74 of Gal4, VP16 (see above) and the ligand-binding domain of a rat GR. The MCS is preceded by a promoter containing six copies of the Gal4 UAS and a minimal CaMV 35S promoter (Aoyama and Chua, 1997).

To construct GVG, the ligand-binding domain of a rat GR was added to the carboxyl terminus of a Gal4-VP16 fusion (residues 1–74 of Gal4 fused to VP16 and residues 519–795 of rat GR). In common with other steroid receptors, the ligand-binding domain of GR interacts in a steroid-sensitive fashion with a cytosolic protein complex involving heat shock proteins 90 (HSP90). (Picard, 1993). The HSP90 complex prevents the bound transcription factor from interacting with essential partners such as the nuclear import machinery or the transcriptional apparatus, but the interaction is disrupted when the steroid receptor domain binds its ligand. As many animal steroids appear to be absent in plants, their receptors have been used in a number of inducible expression systems. This system has been successfully used in a great number of studies (over 50 so far) in Arabidopsis, making it by far the most widely adopted system to date. Most studies have been done in sterile culture but dexamethasone can be taken up from the soil as indicated by the partial complementation of *tps* (Trehalose-6-Phosphate Synthase 1) by UBIQUITIN10>GR>TPS using the GVG system (van Dijken et al., 2004). All the induction characteristics of GVG in Arabidopsis are approximately 10-fold better than those reported for GVG in tobacco (Aoyama, 1998; Aoyama and Chua, 1997). The

higher induction ratio in Arabidopsis may be attributable to lower uninduced expression levels in this species relative to tobacco (Aoyama, 1998). Despite its extensive use in Arabidopsis, it is probably not the ideal system for this species, as it has been observed that the GVG molecule often perturbs Arabidopsis development and triggers defence responses when it is activated by dexamethasone (Kang et al., 1999; Zuo and Chua, 2000; Zuo et al., 2000). A promising alternative for use with genes under control of the Gal4-UAS is based on the ecdysone receptor (EcR) from the spruce budworm (*Choristoneura fumiferana*) and a non-steroidal ecdysone analogue methoxyfenozide (Koo et al., 2004; Padidam et al., 2003). Padidam et al. (2003) developed this receptor domain and methoxyfenozide as a regulatory module that has the potential for field application, but it is applicable in the laboratory and greenhouse too. The composition of the VGE system is detailed below

Figure 3. The transcriptional activator VGE (Koo et al., 2004; Padidam et al., 2003) consists of the VP16 activation domain, Gal4 DNA-binding domain (1–147) and the ligand-binding domain of Choristoneura fumiferana ecdysone receptor (EcR; residues 206–539). Expression is started from the Cassava mosaic virus promoter (CsV). The CgCP gene was inserted downstream of the target promoter containing five copies of the Gal4 UAS.

VGE and GVE are expressed from the Cassava mosaic virus promoter on the same T-DNA as the Gal4-UAS cassette into which genes are cloned. For example, luciferase reporter expression assays indicated an average 10^3-fold induction in GVE while quantitative Northern and Western blots using VGE show an average 700-fold induction with undetectable background expression in most transformants. According to Padidam et al. (2003) and Koo et al. (2004) VGE requires three times more methoxyfenoxide for 100% induction than GVE. Both systems respond quite rapidly upon induction (by soil drenching, induction within 6-12 h), induced expression can be detected by western blotting in all major organs within 120 h. Other methods of methoxyfenoxide application are germination of plants on agar media containing the inducer or induction of leaf discs, whereas foliar application is ineffective which denies the possibility of local induction (Padidam, 2003). So far, no negative effects on Arabidopsis growth by induction conditions have been observed or published.

GVG and VGE in other species

Originally used in tobacco (Aoyama and Chua, 1997), this dexamethasone-inducible system has also been successfully used in BY-2 cell culture system (Nara et al., 2000; Yang et al., 2005). In other dicots this system has been tested with mixed and limited success. In lettuce, it proved possible to regulate the ipt gene such that transformants could be selected on cytokinin-free medium without recourse to antibiotic resistance (Kunkel et al., 1999). However, few other details of the expression characteristics of GVG in this species are known. Andersen et al. (2003) found that GVG induced developmental abnormalities in L. japonicus and this precluded the interpretation of transgene phenotypes. Developmental abnormalities were also observed in rice (Ouwerkerk et al., 2001) after 2 weeks when GVG was fully induced, but were attributed to high concentration of dexamethasone and long induction time. Indeed, Lee et al. (2003) obtained a cyclin overexpression phenotype after 7 days apparently without seeing abnormalities in vector-only controls that expressed as much GVG. However, in all these lines there was very significant uninduced expression. Wong et al. (2004) obtained very rapid induction of target transcripts from endogenous levels in rice cell cultures, but the influence of the expression system on the cells was not investigated. Ouwerkerk et al. (2001) modified the original GVG vector to make it suitable for rice transformation and for the easy insertion of target genes and promoters in polylinker sites downstream of UAS and upstream of GVG, respectively. The GVG system has been tested also in two gymnosperm species, *Pinus taeda* and *Pinus virginiana*. In P. taeda cell cultures, 100-fold induction of GFP expression was achieved with 5 to 10 µM dexamethasone (50% maximum at 0.5 µM; Tang and Newton, 2004a). However, in P. virginiana cell cultures, the system was induced only 4- to 8-fold and (Tang and Newton, 2004b).

1.2.4. Chemically inducible expression from the O_{lexA}-46 promotor (XVE) in *Arabidposis*

The O_{lexA}-46 promoter is the target for the XVE transcription factor, which is activated by oestrogens (Zuo et al., 2000). The XVE protein comprises residues 1–87 of the E. coli lexA repressor domain fused to the VP16 transcription activation domain and the

ligand-binding domain (residues 282–595) of the human oestrogen receptor. In the presence of oestrogens such as 17-ß-oestradiol or 4-hydroxyl tamoxifen, this protein binds to eight copies of the lexA operator in OlexA-46 and activates transcription from the adjacent CaMV 35S minimal promotor (Zuo et al., 2000). The XVE transcription factor is expressed from the strong synthetic promoter G10-90 that exhibits eightfold higher activity than CaMV 35S (Ishige et al., 1999). The vector pER8 carries the G10-90::XVE cassette on the same T-DNA as the OlexA-46 promoter (Figure 4)

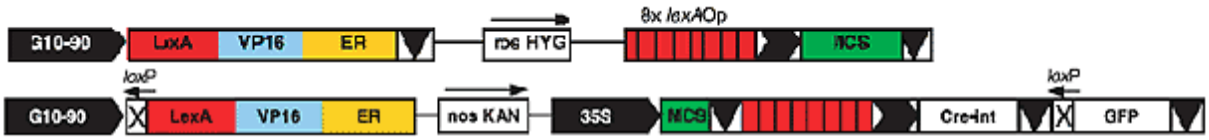

Figure 4. *above:* pER8 (pER10 is identical to pER8 except that the selectable marker confers resistance to kanamycin rather than hygromycin). Promoter G10-90, a synthetic promoter (Ishige et al., 1999) controlling XVE; XVE, DNA sequences encoding a chimeric transcription factor containing the DNA-binding domain of lexA (residues 1–87), the transcription activation domain of VP16 (residues 403–479) and the regulatory region of the human oestrogen receptor (residues 282–595); 8x lexA Op, eight copies of the lexA operator sequence upstream of a minimal promoter.
Below: pX8-GFP for inducible conditional knockout studies. A gene of interest in the MCS is expressed from the CaMV 35S promoter. Oestrogen application causes Cre mediated excision of this gene plus the selectable marker and the XVE elements causing GFP to be expressed from the G10-90 promoter. If the construct is introduced into a background that is null for the gene of interest, the mutant phenotype will be permanently expressed in the GFP-positive sectors (Zuo et al., 2006).

Absolute expression levels and the fold induction are difficult to compare with other systems as the system has been characterized using GFP, Northerns and RT-PCR that generate relative expression values and do not sensitively quantify uninduced levels. However, these methods suggest that genes of interest can be induced significantly more than 100-fold from undetectable or low levels (Carranco et al., 2004; Zuo et al., 2000). For similar reasons, it is difficult to evaluate the stringency with which XVE is regulated as we are not aware of the system being used with sensitive physiological reporters such as bacterial *ipt* or *avr* genes. However, an activation-tagged line that carries the O_{lexA}-46 promoter 326-bp upstream of the Arabidopsis AtIPT8 locus generated cytokinin over accumulation phenotypes on 17-ß-oestradiol but was apparently normal in the absence of inducer (Sun et al., 2003). Quantification of Northern blots indicated that 5 µM 17-ß-oestradiol is sufficient for maximal induction, while 50% induction is achieved with 1 µM (Zuo et al., 2000). Oestrogens such as 17-ß-oestradiol are efficiently taken up by seedlings from culture

medium as induced transcripts can be detected within less than 1 h of treatment (Zuo et al., 2000). Accumulation appears to be slower than with other systems as transcripts are most abundant only 24 h after induction and they decrease again over the next 3 days indicating that 17-ß-oestradiol is unstable under culture conditions and in planta (Guo et al., 2003; Zuo et al., 2000). In order to apply 17-ß-oestradiol it has been added to the culture medium or sprayed onto seedlings, or in the greenhouse sprayed, painted or infiltrated, whereas application via the soil is not reported so far. Due to the relatively low volatility of 17-ß-oestradiol as well as the slow transport in plant tissues (Tornero et al., 2002) it is well suited for localized expression. In contrast, although dexamethasone remains confined near to painted areas of mature leaves over 24 h (Craft et al., 2005), it is systemically transported via the roots throughout the shoot so may not be so suitable for sustained localized expression. Zuo et al. (2006) report that 17-ß-oestradiol has never been observed to elicit physiological effects on plants and that there is no evidence that it disturbs endogenous gene expression or development in Arabidopsis. Although lexA operator sequences (CTGTWWWWWWWWACAG) occur several thousand times in plant genomes, there are no reports of developmental abnormalities arising through use of XVE or oestrogens in plants. Furthermore, Zuo et al. (2000) demonstrate that this system does not induce PR (Pathogenesis related) proteins as GVG does. The lexA operator contains two sites that are prone to cytosine methylation in plants and, although it is unclear whether this would compromise binding, the empirical evidence is that the XVE system is stably expressed (Zuo et al., 2000).
This system has been used in a dozen different studies in Arabidopsis to successfully induce the expression of proteins or RNAi molecules and for inducible activation tagging.

XVE in other species

The XVE system has been used successfully following transient expression in tomato leaves (Pedley and Martin, 2004). It has also been used in rice to drive expression of the Cre recombinase in the CLX system in order to excise a selectable marker after regeneration of transformants (Sreekala et al., 2005). Expression in monocots is improved by using the rice *Actin1* promoter in place of G10-90 (Zuo et al., 2006).

1.2.5. The pOp/LhG4 Inducible System: an *E. coli* lac operon derivate

Chemically inducible pOp System in Arabidopsis

The pOp/LhG4 system is originally used as a transactivation system, but has been modified to facilitate chemically inducible expression (also see Figure 5) and has been characterized in tobacco and Arabidopsis (Craft et al., 2005; Samalova et al., 2005). It is composed as follows:

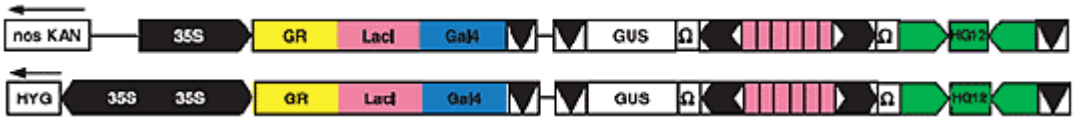

Figure 5. pOpOff2^{Kan} and pOpOff2^{Hyg} with a CaMV 35S-LhGR cassette on the same T-DNA as the target and reporter loci with either kanamycin or hygromycin resistance markers (Wielopolska et al., 2005).

The principal design of the system is based on the *E. coli lac* operon. It contains the artificial promoter 'pOp' and a chimaeric transcription factor 'LhG4 (Moore et al., 1998).The pOp promoter consists of two ideal *lac* operators positioned upstream of a minimal CaMV 35S promoter (-50 to +8 relative to the transcription initiation point) and is physiologically silent in the absence of the artificial transcription factor LhG4 (Baroux et al., 2001; Craft et al., 2005; Lexa et al., 2002; Samalova et al., 2005) LhG4 binds to the lac operators in the pOp promoter via its DNA-binding domain, which is derived from a high-affinity DNA-binding mutant of the E. coli lac repressor (Lehming et al., 1987). Once bound, LhG4 promotes transcription from the minimal CaMV 35S promoter of pOp by virtue of a transcription-activation domain derived from the Saccharomyces cerevisiae transcription factor Gal4p (Moore et al., 1998). The sequences that are critical for transcriptional activation in Arabidopsis lie within the carboxy-terminal 17 amino acids of the Gal4 activation domain and conform to the consensus for eukaryotic acidic activation domains (Rutherford et al., 2005). To generate a chemically inducible system where all components are on one vector LhG4 was fused to the ligand-binding domain of a rat GR (Craft et al., 2005; Schena et al., 1991). The resulting dexamethasone-inducible transcription factor LhGR has so far been used for the inducible expression of a cytokinin biosynthetic gene (Craft et al., 2005; Jasinski et al., 2005), RNAi molecules (Reddy and Meyerowitz, 2005; Wielopolska et al., 2005) and transcription factors protein kinases or dominant-

inhibitory mutant proteins (Miltos Tsiantis and Jeff Leung, unpublished data cited: Moore et al., 2006 after personal communication; H. Betts and I. Moore, University of Oxford, UK, unpublished data). Under greenhouse conditions an average 10^3 fold-induction and induced GUS activities of 200 pmol min-1 µg-1 in seedlings and mature leaves can be found, so the induced GUS activity is fourfold higher than average CaMV 35S-GUS levels.

The pH-TOP vector, which additionally incorporates the TMV O translation enhancer in the 5'UTR, can achieve more than 10-fold higher GUS activities and, although it also exhibited higher uninduced activities than pOp or pOp6, the dynamic range was still at least 10^3-fold.

Induction is rapid in cultured seedlings with GUS activities beginning to increase after 1 h of dexamethasone application and proteins visible by Western blot within 4 h (Jeff Leung, ISV, Gif sur Yvette, France, cited Moore et al., 2006 after personal communication). Under greenhouse conditions, considerable GUS activity was detected in leaves at 6 h after subterranean irrigation with dexamethasone, so uptake via the roots and transport to the shoots is also rapid. After 12 to 24 h of induction, GUS staining throughout the greenhouse-grown plants resembled that of constitutive CaMV 35S_GUS lines. In contrast to tobacco, where the required dexamethasone concentration required for induction is 100-fold lower than in Arabidopsis, the inducer seems to be metabolized or compartmentalized more rapidly in Arabdopsis (Wielopolska et al., 2005; Aoyama and Chua, 1997).

Chemically inducible pOp System in other species

Samalova et al. (2005) have characterized the pOp6/LhGR system in tobacco using GUS, luciferase and ipt reporter constructs. As with Arabidopsis, the best induction characteristics were obtained with the newer pOp6 promoters. In the absence of dexamethasone, GUS activity was indistinguishable from background and CaMV 35S>GR>ipt exhibited normal root growth rates and normal morphology throughout their life-cycle (Samalova et al., 2005). Nevertheless, induction of *CaMV 35S>GR>ipt* lines by germination on inductive medium or by a single application of dexamethasone to soil-grown plants resulted in more severe cytokinin overproduction phenotypes than those previously reported with other inducible systems in tobacco (Böhner and Gatz, 2001; Faiss et al., 1997; McKenzie et al., 1998) and in many

cases the plants died (Samalova et al., 2005). Interestingly the pOp6/LhGR system is definitely more sensitive to dexamehasone in tobacco than in Arabidopsis (Moore et al., 2006). Dexamethasone does not affect tobacco growth up to at least 60 µM in soil water (Samalova et al., 2005). The system has not been tested with the BY-2 suspension culture.
The older pOp/LhGR system has been tested in potato, but induced GUS activities were low (J. Craft, A. Martinez, I. Jepson and I. Moore, University of Oxford, UK, unpublished data, cited from Moore et al, 2006), but it has not been tested with the newer pOp6 promotor.

1.3. Heat shock-based inducible systems

Inducible systems, both chemically and environmental stress dependent are found in all living organisms. One example for environmental stress dependent gene induction is heat shock activation. This is a highly conserved response to cellular stress. Heat shock proteins, which function as chaperonins, help to compensate for heat damages on cellular level (Beckmann et al., 1992). The activation of the heat shock response is regulated at the transcriptional level (Morimoto et al., 1992), and heat shock elements (HSE), short sequences present in all HSP promoters have been identified to be essential for stress inducibility (Bienz and Pelham, 1987). HSEs contain multiple copies of the five base pairs sequence NGAAN (Amin et al., 1988), detailed mutational analysis identified AGAAC as the optimal sequence (Cunniff and Morgan, 1993), whereas the number of pentameric units in a HSE can vary, but a minimum of three is required for efficient heat-inducible expression (Fernandes et al., 1994). When positioned upstream of a heterologous promoter, HSEs can confer heat stress inducibility to that promotor (Bienz and Pelham, 1986).

1.3.1. Heat shock inducible systems in animals

Heat shock promoters have extensively been used in different experimental systems. The highly conserved nature of heat stress response allows the use of heterologous promoters. Thus, Xenopus and mouse HSP70 promoters were first tested in the fish

system (Adam et al., 2000), later followed by experiments with fish promoters (Halloran et al., 2000; Molina et al., 2000; Scheer et al., 2002).

1.3.2. Heat shock-inducible systems in plants

Plants are sessile organisms with outstanding abilities to withstand temperature stresses. Gene expression in plants can therefore be induced by a mild heat-shock with minor impact on the fitness, using expression cassettes driven by heat-regulated promoters (Ainley and Key, 1990; Schöffl et al., 1989). Plant heat-inducible promoters are well known for their strong, rapid and short lasting transcriptional reponse to heat stress (Vierling and Kimpel, 1992; Nover et al., 2001) The plant promoters from the small heat shock protein (sHSP) gene family provide highly sensitive responses to mild temperature variations (Sun et al., 2002). Thus, in *Nicotiana tabacum* the promotor *hsp17.6L* from soybean can control antibiotic resistance in a temperature-dependent manner (Severin and Schoffl, 1990) as well as the expression of the FLP recombinase in maize (Lyznik et al., 1995) and *Arabidopsis thaliana* (Kilby et al. 2000). Similarly, the *A. thaliana hsp18.2* promotor was successfully used as a heat-inducible expression system in tobacco BY2 cells (Yoshida et al., 1995), and to achieve heat-inducible RNAi expression in *A. thaliana* (Maslcaux et al., 2004). Another heat-shock inducible system has been tested in *Physcomitrella* utilizing the soybean heat-shock Gmhsp17.3B promotor along with ß-glucuronidase (GUS) and F-actin marker (GFP-talin) as reporter genes. In stably transformed moss lines, Gmhsp17.3B-driven GUS expression was extremely low at 25 °C. In contrast, a short non-damaging heat-treatment at 38 °C rapidly induced reporter expression over three orders of magnitude, enabling GUS accumulation and the labelling of F-actin cytoskeleton in all cell types and tissues. Induction levels were tightly proportional to the temperature and duration of the heat treatment. Treatment with acetyl salicylic acid (ASA) and benzyl alcohol (BA, a membrane fluidiser) also induced GUS expression at 25 °C (Saidi et al., 2005). As an example that other factors can induce

Pollen embryogenesis is a very useful tool to study the molecular mechanisms of early plant embryogenesis, since the cells used are freshly isolated single cells, the microspores or mid-bicellular pollen, embryogenesis occurs without an intervening callus phase and no addition of growth regulators is required to induce and maintain embryogenesis. (Pechan et al. 1991;Vicente, Benito Moreno and Heberle-Bors

1991). In the two best characterized pollen embryogenesis systems, i.e. those in *Brassica napus* and *Nicotiana tabacum*, stress treatment is sufficient to switch normal gametophytic development towards embryogenesis. This stress treatment includes short heat stress and/or starvation. During stress treatment the expression of as yet only partly characterized mRNA and protein species, some of which probably correspond to products of heat shock genes (Pechan et al. 1991). For example, induction of pollen embryogenesis by an *in vitro* starvation treatment of mid-bicellular tobacco pollen was accompanied by a dramatic increase in the levels of the *Nthsp 18p* transcript, which accumulated in embryogenic pollen grains at 25°C; lower levels of this transcript were also detected in early pollen-derived embryos. A similar pattern of expression was observed in transgenic tobacco plants containing the promoter of *Gmhsp 17•3-B*, a soy-bean homologue of the *Nthsp 18P* gene, fused to the GUS reporter gene (Zarsky et al. 1995).

These heat shock genes (HSPs) may be required for the microspores to survive the heat treatment, and the activation of hsp genes could simply reflect a normal stress response, as in other plant tissues. But it is also possible that specific heat shock proteins are directly associated with the process of induction of pollen embryogenesis (Zarsky et al. 1995). The second possibility seems more likely since it is known that many heat shock genes, in animals as well as in plants, are not only induced by heat or sometimes by other stress factors, but also developmentally regulated, primarily during gametogenesis and embryogenesis (Nagao et al. 1986; Bensaude, Mezger and Morange, 1991; Zimmerman and Cohill 1991). An increasing amount of evidence suggests that in addition to the defense function during thermal shock, the HSPs have an important function in cell proliferation and differentiation (Bond et al. 1987). Both the expression of HSPs in the absence of heat shock and the altered response to high temperature at different developmental stages indicate a possible involvement of HSPs in normal development.

1.4. Heat shock enhancer element as mediator in gene expression.

A very promising inducible system bases on heat shock induction was developed from the group of Thomas Czerny. This inducible element consists of multimers of eight idealized heat shock factor (HSF) binding sites, called heat shock elements

(HSE). As already mentioned above, HSEs can confer heat inducibility to close-by heterologous promoters. When introduced into zebrafish, this element showed superior properties in comparison to natural heat shock promoters because of its artificial structure: dramatically reduced background activity, improved inducibility and loss of all tissue specific components (Bajoghli et al., 2004).

2. Aims of the thesis

In search for an alternative to chemically inducible and the existing heat inducible systems in plants a heat shock element from zebrafish (Bajoghli et al., 2004, see also chapter 1.4.) is to be tested in *Arabidopsis thaliana* as a plant model system.
The idealized HSE multimer from zebrafish has been introduced into a binary shuttle vector in front of a GUS reporter gene under control of a CaMV minimal promoter. To achieve this, the GUS gene under control of a CaMV minimal promoter from *p221.9 Gre6* was used to replace the same gene on *pBI101.1* (under full CaMV promoter) to obtain a reporter GUS gene genetically silent when not enhanced by an extrernal factor such as a heat shock element. For the final test construct, the heat shock element from *pSGH2* was introduced directly before the minimal promoter of the reporter GUS gene. This construct was then transformed into *Agrobacterium tumefaciens* by electroporation. Cultures from positive transformants (screened by antibiotic selection and reverse ligation) were used for *Arabidopsis* floral dip to transfer the vector into *Arabidopsis* plants. The transgenic lines were then tested for their heat shock phenotype, using a quantitative assay to evaluate the magnitude of induction.

3. Materials

3.1. Plasmids

- *pSGH2*: This construct is the source of the 8xHSE sequence, which was cut out using HindIII and SalI.

- *pBI101.1*: pBI101 provided the backbone of the later pBI-HSE-35MinGUS and pBI-35MinGUS constructs prepared for this work.

- *p221.9 Gre6:* The GUS gene under control of a minimum 35S promotor was obtained from this construct, which was cut out using BamHI and EcoRI.

- *pBI-35MinGUS:* The construction of pBI-35MinGUS was essential for both a control construct with minimum GUS activity and as a first step towards pBI-HSE-35MinGUS as a test construct for the heat shock enhancer. A scheme of the plasmid with the most important restriction sites can be found in Appendix, page 67.

- *pBI-HSE-35MinGUS:* To test the Heat shock enhancer element, it was cloned in front of the GUS gene under control of the 35S minimal promoter from the pBI-35MinGUS construct. A scheme of the plasmid with the most important restriction sites can be found in Appendix, page 68.

3.2. Media, buffers and solutions

Media

Media	Purpose	Total Volume	pH adjusted to	Component	Amount of component
LB	Bacterial Growth	1000 ml	7,4	NaCl	10 g
				Bactotrypton	10 g
				Yeast extract	5 g
				Bacterial Agar	15 g if required
CPY	Bacterial Growth	1000 ml	7,2	Yeast extract	1 g
				Bactotrypton	5 g
				Sucrose	5 g
				$MgSO_4.7H_2O$	0,493 mg
				Bacterial Agar	20 g if required
SOB	Bacterial Transformation	750 ml	6,7-7,0	Bactotrypton	15 g
				Yeast extract	3,75 g
				NaCl	0,4388
				KCl	0,14
				$MgCl_2.6H_2O$	1,525 g
				$MgSO_4.7H_2O$	1,847 g

Buffers

Buffer	Purpose	Total Volume	pH adjusted to	Component	Amount/concentration of component
TB	Bacterial Transformation	750 ml	6,7	PIPES	1,785 g
				MnCl2	8,71 g
				$CaCl_2.2H_2O$	1,65 g
				KCl	13,97 g

CTAB	Plant DNA isolation	100 ml	8,0	CTAB	2 g
				NaCl	8,182 g
				EDTA	0,585 g
				Tris/HCl	1,212 g
GUS Staining solution	Histochemical staining	1 ml	7,0	$NaPO_4$ Buffer pH7 (see below)	0,1 M
				EDTA	10 mM
				Triton X-100	0,10%
				X-Gluc	2mM
$NaPO_4$ Buffer pH7	Stock Solution for GUS staining	10 ml	7,0	NaH_2PO_4 1M Stock Solution	0,390 ml
				Na_2HPO_4 1 M Stock Solution	0,610 ml
				H_2O	9 ml
GUS Extraction Buffer	Plant Protein extraction	1000 ml	7,0	$NaPO_4$ Buffer pH7 (see above)	50 mM
				EDTA	10 mM
				SDS	0,10%
				Triton X-100	0,10%
				ß-Mercaptoethanol	10 mM
4-MUG Reaction Solution	Fluorescent GUS Assay	25 ml	7,0	4-MUG	0,0116 g
				GUS Extraction Buffer (see above)	25 ml
4-MU Standard Stock solution	Fluorescent GUS Assay	1 ml	7,0	4-MU	0,0176 g
				Methanol	1 ml
HEPES	Agrobacterium transformation	100 ml	7,0	HEPES	23,8306 mg

Standards: a standard array has been established by diluting the stock solution and after that each standard by 1:100 (10 µl stock or standard and 990 µl) of GUS extraction buffer

Oligonucleotides

Name	Sequence	Numbers of nucleotides				GC content (%)	Calculated Melting Temperature (°C)
		A	T	G	C		
Primer GUS 5'	GGCCTGTGGGCATTCAGTCTGGATC	3	7	9	6	60	58,5
Primer GUS 3'	CGGCCTTAGGTAGCGTCGCATTACG	4	6	8	7	60	54,12

3.3. Plant Material

- *Arabidopsis thaliana, Columbia*

3.4. Bacteria

- *Escherichia coli: DH5a strain*

- *Agrobacterium tumefaciens: Strain AGL1*

3.5. Chemicals

All chemicals used were of p. A. (pro Analysii) grade or higher.

Category	Purpose	Compound Full Name	Compond Short Name/Formula
Antibiotics	Bacterial selection	Ampicilin	Amp
	Bacterial and Plant selection	Kanamycin	Kann
	Plant selection	Hygromycin	Hyg
Basic chemicals	Media and Buffer	Sodium Chloride	NaCl
		Bactotrypton	
		Yeast Extract	
		Potasium Hydroxide	KOH
		Magnesium Sulfate Heptahydrate	$MgSO_4.7H_2O$
		Potasium Chloride	KCl
		Magnesium Chloride Hexahydrate	$MgCl_2.6H_2O$
		1,4-Piperazinediethanesulfonicacid	PIPES
		Manganese (II) Chloride	$MnCl_2$
		Calcium Chloride Dihydrate	$CaCl_2.2H_2O$
		Ethylendiamintetraacetat, Sodium salt	EDTA
	Media and Buffer, Plant Growth and Transformation	Sucrose	$C_{12}H_{22}O_{11}$
	Plant Transformation	Silwett I-77	

3.6. Enzymes and Buffers

- *Restriction enzymes:* HindIII, SalI, BamHI, EcoRI from Fermentas, together with suitable buffers suggested by Fermentas Five Buffer System table
- *Ligases:* T4 DNA Ligase from Fermentas and 10X T4 DNA Ligase Buffer

- *DNA Polymerases:* GoTaq® Hot Start DNA Polymerase and Green Buffer including loading buffer for Agarose Gels, together with $MgCl_2$ and dNTP solutions

3.7. Kits

- *Promega Wizard® Plus SV Minipreps DNA Purification System*
- *Quiagen Plasmid Midi Kit*
- *QIAprep Spin Miniprep Kit*
- *Promega Wizard® SV Gel and PCR Clean-Up System*

3.8. Other Equipment

- *Tubes, Pipette Tips of various sizes and other plastic equipment from Eppendorf*
- Victor^{2}D Fluorometer

4. Methods

4.1. Molecular Biology methods

4.1.1. Transformation of *Escherichia coli*

Preparation of Ultra-competent cells (Inoue Method)

To increase the chance of DNA uptake into E. coli (competence of cells) the cells of the chosen strain (DHa) need treatment including cold and starvation stress.

Inoculate 100 ml of SOB from LB overnight culture and grow to an optical density (OD) of 0,4 at 28°C then change to room temperature and grow there to an OD of 0,6. Place cell suspension on ice for 10 minutes and then centrifuge at 2500xg (5000 rpm in GSA rotor) for 10 minutes at 4°C. Discard supernatant and resuspend cell pellet in 32 ml chilled TB. Incubate on ice for 10minutes. Centrifuge again under the same conditions, discard the supernatant and resuspend the pellet in 8 ml TB and add 0,56 ml DMSO. Incubate again for 10 minutes on ice. Then place 100 ?l aliquots in tubes and freeze in liquid N_2. Store the tubes at –80°C until used for transformation.

Transformation of Ultra-competent cells

Thaw one tube of prepared cells per transformation event on ice and add about 1 ?l of DNA solution (dependent on DNA concentration, as high amounts of DNA reduce transformation efficiency) and stir gently with the pipette tip. Incubate on ice for 30 minutes and then place into a thermo block at 42°C for 40 seconds for heat shock and immediately place the tubes back on ice. Add 1 ml of chilled LB, seal the tube with parafilm and incubate at 37°C for 1 hour on a shaker. After incubation take 100 ?l of cell suspension and put it onto LB plates containing the appropriate antibiotic as selection
marker (dependent on used vector, in this case Ampicillin and Kanamycin). Centrifuge the remaining suspension and remove the majority of the supernatant. Resuspend the pellet in the remaining liquid and put it onto other LB antibiotic plates.

Incubate the plates overnight at 37°C. From received colonies pick single ones and streak out onto a fresh LB antibiotic plate (further called master plate) and incubate again overnight at 37°C. Cells from that plate are used for isolation of transformed plasmid in different amounts and for preparation of Glycerol stocks.

4.1.2. Transformation of *Agrobacterium tumefaciens*

Preparation of competent cells

Agrobacterium tumefaciens AGL1 cells from a Glycerol stock were inoculated into 2 ml CPY medium (instead of LB as recommended in the used protocol, cells tend to grow in big clumps in LB, but not in CPY) and grown overnight at 28°C. This culture was inoculated into 5 ml CPY and grown on a shaker at 28°C to an optical density (at 600 nm) of 1 to 1,5. The culture was then chilled on ice for 15 minutes, spun down at 5000 rpm for 20 minutes at 4°C. The medium was removed and discarded, the pellet washed three times (using the same centrifugation settings as above) with 10 ml 1mM HEPES (2-(4-(2-Hydroxyethyl)-1-piperazinyl)-ethansulfonic acid) at pH 7.0. After washing, the pellet was resuspended in 540 µl of sterile 10% glycerol, divided into 45 µl aliquots in special cryo tubes frozen with liquid nitrogen and stored at -80°C for transformation.

Transformation of competent cells by electroporation

For each one transformation a tube of previously prepared Agrobacterium tumefaciens AGL1 competent cells was thawn on ice, 1 µl plasmid solution (containing about 0,2 µg DNA) was added and the mixture transferred to a prechilled electroporation cuvette (0,2 cm gap). The electric pulse applied for transformation was at field strength of 12,5kV/cm for 5 ms at 15 µF. Directly after the pulse 1 ml of CPY was added and incubated at 28°C for 1 hour. After incubation take 100 ?l of cell suspension and put it onto LB plates containing the appropriate antibiotic as selection marker (dependent on used vector, in this case Ampicillin and Kanamycin). Centrifuge the remaining suspension and remove the majority of the supernatant. Resuspend the pellet in the remaining liquid and put it onto other LB antibiotic plates.

Incubate the plates overnight at 28°C. From received colonies pick single ones and streak out onto a fresh LB antibiotic plate (further called master plate) and incubate again overnight at 28°C. Cells from that plate are used for isolation of transformed plasmid in different amounts and for preparation of Glycerol stocks.

4.1.3. Preparation of Glycerol Stocks

For long time storage of bacterial strains and especially transformants a culture was grown overnight. 1,24 ml of this culture was intensively mixed with 0,5 ml 87% sterile Glycerol inside a special cryotube and immediately dropped into liquid nitrogen for shock freezing. The frozen tubes were stored at -80°C.

4.2. DNA isolation and processing

4.2.1. DNA isolation (CTAB)

To prove successful transformation of Arabidopsis plants DNA was isolated using this protocol. The isolated DNA was used for PCR to check for inserts.
Plant material was grinded in a mortar together with liquid nitrogen. 500 µl of CTAB mix was added and the mixture was put to a shaker for 30 minutes at 60°C. A mixture of 250 µl Phenol, 250 µl Chloroform and Isoamylalcohol (24:1) was added, mixed and separated in a centrifuge for 5 minutes at 1600 g. The water phase was transferred to a new tube, shortly shaken with 500 µl of a mixture of Chloroform:Isoamylalcohol (24:1) and separated again in a centrifuge for 5 minutes at 1600 g. The water phase was transferred to a new chilled tube, 500 µl of prechilled (4°C) Isopropanol was added. The mixture was then spinned down in a centrifuge for 15 minutes at 18300 g at 4°C. The remaining liquid was slowly removed and the DNA pellet was carefully washed with 200 µl of prechilled 70% Ethanol. After a final centrifugation for 1 minute at 18300 g at 4°C the remaining liquid was carefully removed, the pellet was dried by leaving the open tube upside down on a paper towel for 5 minutes. The dried pellet was dissolved in 50 µl TB buffer for PCR.

4.2.2. Plasmid isolation (Miniprep)

Lower purity (Promega kit)

In order to obtain plasmid DNA material in amounts suitable for most further processing steps, isolation of plasmids from overnight cultures of transformed bacteria is essential.
Isolation was done in accordance to the manufacturer's manual. (Promega Wizard® Plus SV Minipreps DNA Purification System)

Higher purity (Qiagen kit)

In case of a single isolation and purification step this method was used in order to obtain DNA of higher purity, which was the case for pBI-35Min-GUS before restriction digest.
Isolation was done in accordance to the manufacturer's manual. (Qiagen plasmid purification handbook)

4.2.3. Plasmid isolation (Midiprep)

When high amounts of plasmid DNA are required (for example serial experiments as particle bombardment), a larger volume of overnight culture (about 100 ml) is used for isolation, which is in its basic steps the same as the Miniprep but uses another purification column.

Isolation was done in accordance to the manufacturers manual. (Qiagen plasmid purification handbook)

4.3. Cloning of the reporter gene and the heat shock enhancer

The overall plan to test the Heat shock enhancer is to place it in front of a ß-Glucuronidase gene (GUS) under a Cauliflower mosaic virus minimal promotor. The first step was to cut out the minimal promotor CaMV –GUS from p221.9 using

BamHI/EcoRI and ligate it into the vector backbone of pBI101.1, resulting in pBI-35MinGUS. Restriction digests, eluation and purification from agarose gels and ligation steps are described below.
The next step was the insertion of the Heat shock enhancer upstream of the reporter gene (GUS). It was first cut out of pSGH2 using HindIII/SalI, the fragment containing the heat shock enhancer was precipitated due to its small size and ligated into pBI-35MinGUS. Precipitation of DNA is described below.

4.3.1. Enzymatic Restriction digest

The cutting of DNA using restriction enzymes can be used for many purposes, such as cloning or identification of a certain plasmid by its fragment size.
For majority of digests Fermentas enzymes were used, together with the patented Buffer system, in an onset size of 20 ?l in total and an incubation time of about 1 hour. Enzyme concentration was never above 1/10 of the onset volume, so in most cases 2 ?l. Higher onsets were also done, for example 50 ?l or 100 ?l by simply using proportional larger amounts of enzyme, buffer, etc. and longer incubation time (several hours to overnight).

4.3.2. Separation of Fragments and Purification of Gel slices

Often Bands on agarose gels contain DNA that are required for further steps (for example fragments of restriction digests) and have to be eluted and purified.
Gels were developed as appropriate (usually 0,8%, only when separating very small fragments 2% gels were used, amperage was usually at 60-80 mA) together with ? DNA marker. The band of the desired size was cut out and purification of DNA was done in accordance with the manufacturers manual (Wizard® SV Gel and PCR Clean-Up System).

4.3.3. DNA Precipitation

When a desired DNA fragment is very small (in this case about 150 bp for the Heat shock enhancer element) instead of agarose gel electrophoresis the DNA is precipitated and dissolved in a suitable buffer for ligation.
From the solution of previous restriction digest 100 µl were mixed with 10 µl of prechilled Sodium acetate (pH=4,9) solution. Then 270 µl of prechilled ethanol (100%) were added, shortly mixed and incubated at -20°C for 30 minutes. The mixture was spun down at 4°C for 15 minutes at 16.000 rpm. The liquid was carefully removed and the DNA pellet washed with 500 µl of prechilled Ethanol (70%). After a final centrifugation at 4°C for 5 minutes at 16.000rpm the liquid was carefully removed, the pellet was dried by leaving the open tube upside down on a paper towel for 5 minutes. The pellet was directly dissolved in Fermentas ligase buffer and used for ligation.

4.3.4. Ligation

The desired DNA fragments previously cut by restriction endonucleases were mixed in a ratio insert to vector backbone of 6:1 together with 10 µl of Ligase aliquote and incubated overnight in a waterbath at 16°C. After the incubation an aliquote of the mix was directly used to transform E. coli competent cells following the Inoue method described above (4.1.1.).

4.4. PCR

Genomic DNA from transgenic *Arabidopsis* plants was used to proof successful transformation using specific primers designed to bind inside of the GUS gene.
A PCR mix was set up as described in the following:
50 µl total per tube

1 µl dNTPs	10 µl GoTaq buffer
1 µl primer GUS 3’	0,2 µl GoTaq Polymerase
1 µl primer GUS 5’	35,8 µl 4x distilled water

The thermocycler used was set to the following program:

94°C	10 min
94°C	1 min 30 sec
58°C	1 min
72°C	1 min
72°C	10 min
4°C	8

4.5. Fluorometric ß-Glucorunidase Assay

Heat shock

Plant explants (leaves and stem) from cultured plants were divided, washed three times with sterile water in petri dishes, divided and put into sterile Eppendorf tubes and treated for two hours with different temperatures (4°C, 17°C, 25°C, 32°C, 37°C and 42°C). The explants were then incubated overnight at 25°C.

Protein isolation and GUS reaction

Explants were transferred into new Eppendorf tubes cooled on ice containing 50 µl of prechilled GUS extraction buffer and a small amount of sterile quartz sand. Each sample was ground using a glass pestle until the liquid was homogenous. The pestle was then rinsed using 150 µ of GUS extraction buffer containing 4-MUG for a final concentration of 1 mmol/l. The tubes were then centrifuged at 13000 rpm and 4°C for 15 minutes. The supernatant of each sample was transferred into a separate new Eppendorf tube, and incubated for 24 hours at 37°C. Before incubation, a Bradford assay was done using the supernatant as samples to evaluate the overall protein concentration of the reaction mix. An aliquote of 1 µl of each sample was added to 800 µl water and 200 µl of Bradford Dye solution and measured using a UV-VIS spectrometer at 595 nm.
To stop the reaction of 4-MUG to 4-MU by ß-glucuronidase 50 µl of 0,1 M Na2CO3 solution were added to an 50 µl aliquote of each sample in a microtiter plate. These

solutions were put into a Victor^{2}D Fluorometer for measurement. Settings were the following:

Exitation wavelength: 355 nm
Emission filter wavelength: 460 nm

4.6. Plant-Related methods

4.6.1. *Arabidopsis* Floral dip transformation

Agrobacterium tumefaciens carrying transgene on a binary vector (pBI-35MinGUS for negative control, pBI-HSE-35MinGUS as test construct for the heat shock element, for details on the two plasmids see Appendix I, page III and IV) were grown overnight at 28°C in CPY medium containing Kanamycin or Hygromycin (Kan for both Heat shock constructs, Hygromycin for GUS positive control plasmid). Cells were spun down and resuspended in 100 ml of freshly prepared 5% Sucrose solution. Before dipping the flowering *Arabidopsis* plants, 1 ml of Silwet L-77 was added to a final concentration of 0,05%. Above-ground parts of plants were dipped in *Agrobacterium* suspension and wrapped into plastic foil. The tip of the foil of each plant was cut off the following morning to avoid fungal growth. Two days after dipping foil was removed from each plant and plants were tied to stacks to give stability and avoid cross-contamination between plant pods. Dipping was repeated once with the same plants after one week to increase transformation efficiency. Plants were watered regularly, and seeds were harvested as soon as seed pods were dried out and brownish.

4.6.2. Seed Sterilisation and Segregation analysis

Seeds taken from dried out (transgenic) plants were washed for 3 minutes in 70% ethanol, for 5 minutes in 50% NaOCl, and afterwards three times in sterile water for one minute each. After removing water from the final washing step seeds were placed onto freshly prepared plates containing MS medium with selective antibiotics

of appropriate concentrations by pipette tip and incubated at 25°C. The plates were checked regularly, occasionally condensation water was removed under sterile conditions, when necessary.
When the seeds finally germinated (after about 2 weeks), the ratio of living (positive transgenic) to dead was determined. Positive transgenics survived through their antibiotic resistance, whereas wild type plants died. The ratio between them depends of the copy number of inserts in the transgenic plants following mendelian inheritance.

4.6.3. ß-Glucorunidase Histochemical Staining

Plant explants (leaves, stem, root and flower) from cultured plants were divided, washed three times with sterile water, put into sterile Petri dishes and treated for two hours with different temperatures (4°C, 17°C, 25°C, 32°C, 37°C and 42°C). Directly after the heat shock the dishes containing the explants were incubated at 25°C for 24 hours. Then the explants were washed once in 50 mM Phosphate Buffer (pH=7) and finally treated with X-Gluc Histochemical staining buffer under vacuum and incubated overnight.
The results were then recorded as specified in chapter 5.4. on page 44.

5. RESULTS

5.1. Cloning of Heat Shock Enhancer into *pBI101.1*

The plasmid construct to test the heat shock enhancer in *Arabidopsis thaliana* was constructed from *pBI101.1*, where the GUS gene under a full CaMV 35S promoter was replaced by the GUS gene under the control of a minimal CaMV35S promotor from *p221.9 Gre6* to reduce background expression of the reporter gene. The heat shock enhancer was then cloned right upstream of the now genetically silent GUS gene under minimal promoter.

Preparation of Fragments

Transformation of basic DNA constructs into E. coli

The three basic plasmids were transformed into *E. coli* DH5a (Inoue Method, see chapter 4.1.1., page 27) and then isolated following Miniprep protocol (see chapter4.2.2., page 30, Promega kit) and transferred to a 0.8% Agarose gel to confirm transformation.

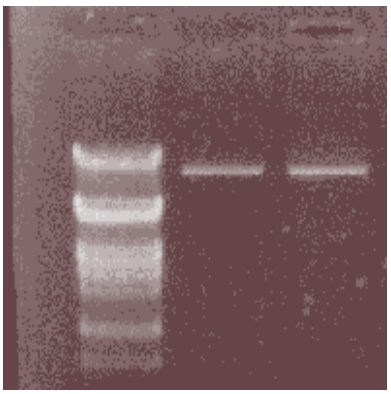

Figure 1: Isolation of pBI101 (02.11.2006)

Figure 2: Isolation of p221 (20.10.2006)

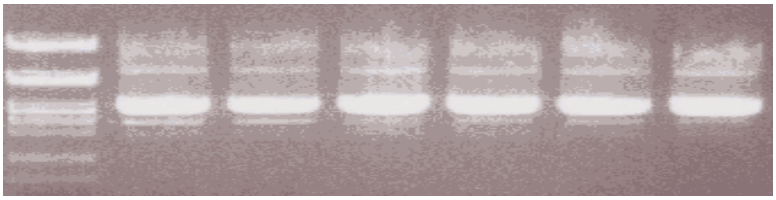

Figure 3: Isolation of pSGH2 (10.11.2006)

Thus, as can be seen on the gel photos, the transformation was successful in every case, though especially p221 needed several repeated transformation attempts. The size of each plasmid matches that of the corresponding band on the gels.

Restriction digests

To obtain the desired fragments needed for ligation, the basic plasmids isolated after successful transformation were cut using suitable restriction enzymes (endonucleases, see chapter 4.3.1., page 31 for details). The resulting fragments were separated on agarose gels (Concentration of agarose dependent of fragment size 0,8% for large fragments, 2% for small ones, such as the eluted fragment of pSGH2 in figure 6).

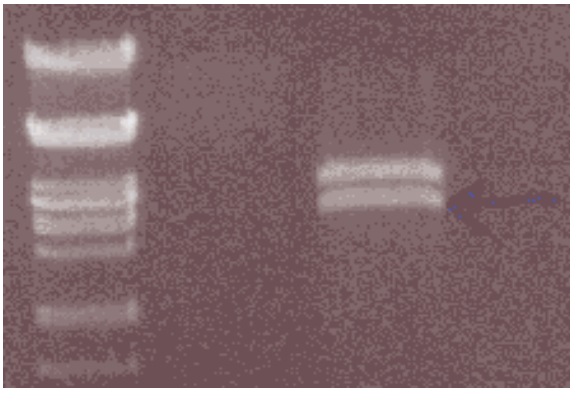

Figure 4: Two fragments of p221 cut with BamH1 and EcoR1. The lower fragment (marked by an arrow) was isolated and purified for the first ligation.

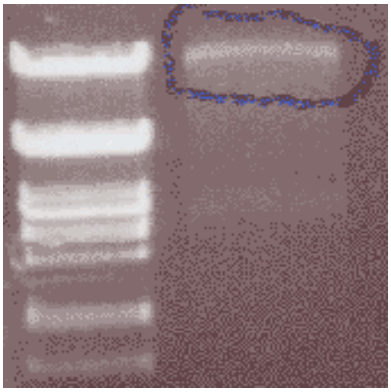

Figure 5: Remaining fragment (plasmid backbone) of pBI101, also cut with BamH1 and EcoR1. This was isolated and purified for the first ligation.

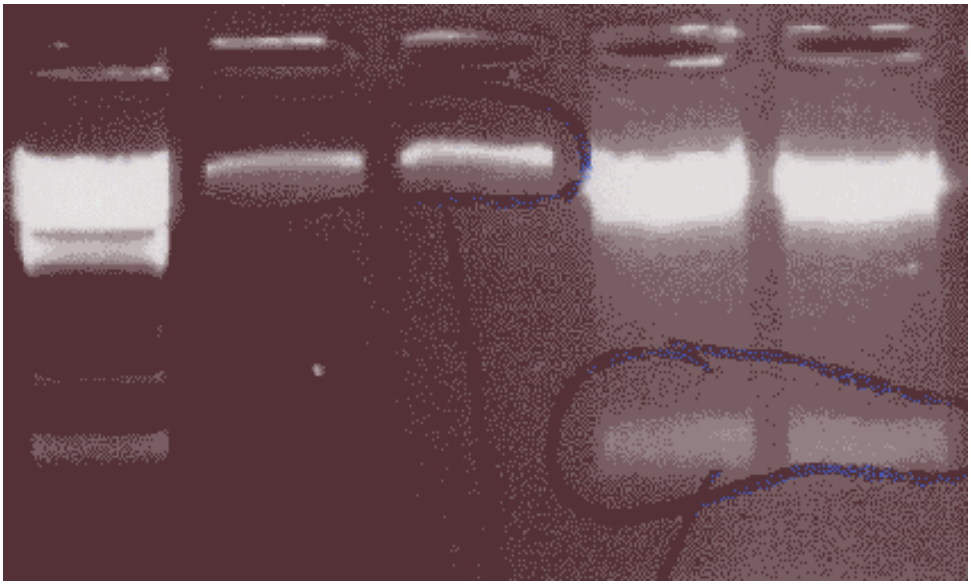

Figure 6: pBI-35S-Min-GUS (Left two lanes) and pSGH2 (Right two lanes) cut with Hind3 Sal1. The small fragment of pSGH2 containing an octamer of HSE and the plasmid backbone of pBI-3S-Min-GUS were isolated, purified and used for the final ligation.

Thus, all digests were successful; the predicted fragment sizes match the corresponding gel bands.

Purification

Fragments from restriction digests were cut out of Agarose gel, purified (see chapter 4.3.2., page 31 for details on Promega Wizard® SV Gel and PCR Clean-up System protocol) and again put on Agarose gels (with 2% Agarose).

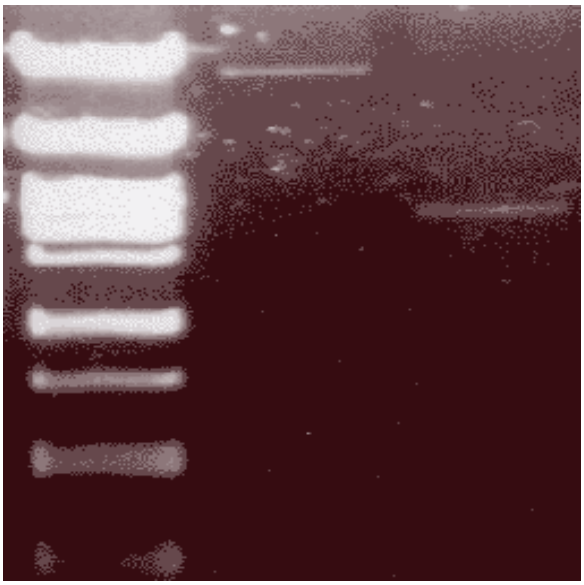

Figure 7: Separation of Fragments after eluation and purification. Left is the plasmid backbone of pBI101, right the 35S Min GUS fragment of p221

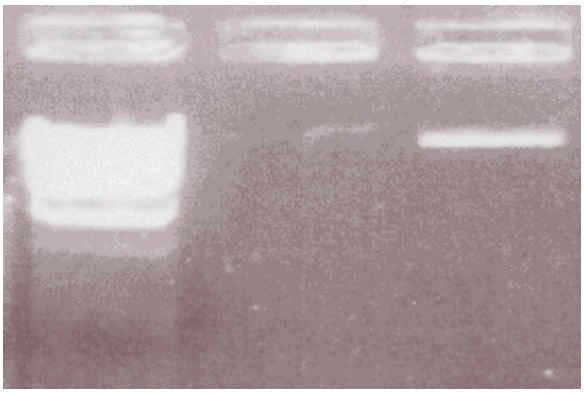

Figure 8: Fragments pBI-35Min-GUS (Backbone, left) and pSGH2 (HSE, right). The gel was run very shortly because of the small size of the pSGH2 fragment.

The purified fragments were still available in sufficient amounts as can be seen on the gel photos as the intensity of the gel bands. The intensity of the gel bands and therefore the amount of DNA decreased naturally because of the purification process.

Ligation

Directly after ligation (incubation overnight at 16°C) an aliquote of the mixture was used to transform E. coli DH5a, which was again incubated overnight at 37°C to allow sufficient growth to allow plasmid isolation (Miniprep) and checking on an Agarose gel (0,8%).

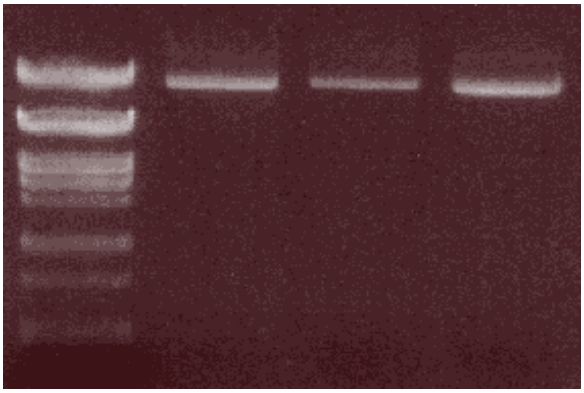

Figure 9: Isolation of three identical plasmids (pBI-35Min-GUS) after ligation.

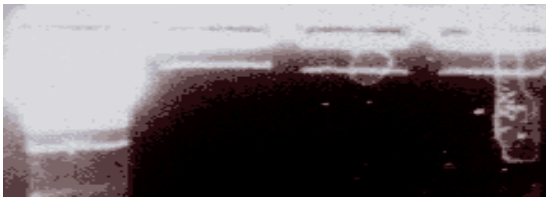

Figure 10: Isolation of three identical plasmids (pBI-HSE-35Min-GUS)

Thus, transformations after the ligation was successful as can be seen on the photos by distinct bands. If the transformed plasmid really contains the desired insert has yet to be determined by reverse ligation (see below).

Reverse Ligation

To prove successful ligation, it is reversed using the same restriction enzymes as for the preparation of the fragments ligated.

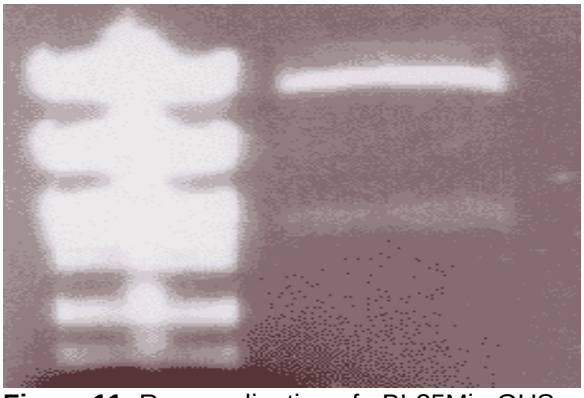

Figure 11: Reverse ligation of pBI-35Min-GUS, using BamH1 and EcoR1.

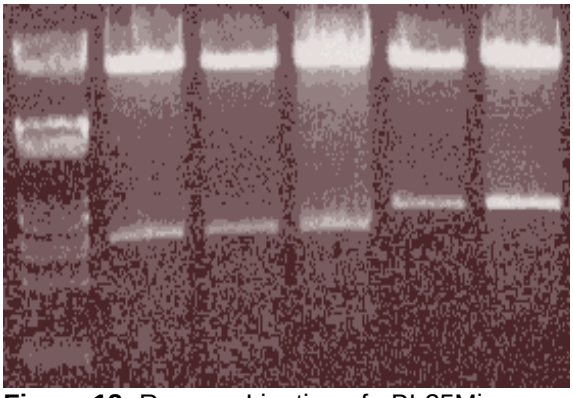

Figure 12: Reverse Ligation of pBI-35Min-GUS (left) and pBI-HSE-35Min-GUS (right),both using Hind3 and EcoR1. The shift in size on the right size indicates the presence of the HSE insert.

Ligation of the minimal promotor GUS from p221 into the backbone of pBI101 was successful, as the treatment with the same restriction enzymes reveals the original fragments before ligation. (**figure 11**). **Figure 12** shows the restriction of both pBI-35MinGUS and the final pBI-HSE-35MinGUS using one restriction enzyme from each previous digests to leave a better prove of the small HSE insert then simply reversing the second ligation. By comparing the sizes of the smaller fragments of both plasmids it is clearly visible that the fragment of pBI-HSE-35MinGUS is about 150 bp larger, therefore containing the desired fragment.

5.2. PCR of *Arabidopsis* Transformants

After transformation plants were screened for positive transformants by PCR. The primers were designed to pair inside the GUS gene (see chapter 3.2. oligonucleotides on page 24)

DNA isolation

The concentration of isolated DNA was measured by UV/VIS Photometry at a wavelength of 260 nm.

Sample	Absorption	Concentration (mg/ml)	Diluted for PCR
I2	0,003	0,146	1:25
I5	0,006	0,317	1:25
I6	0,008	0,403	1:25
I15	0,01	0,488	1:25
I18	0,011	0,537	1:25
I19	0,004	0,208	1:25
I20	0,02	0,977	1:50
I21	0,016	0,781	1:50
G3	0,009	0,439	1:25
M9	0,011	0,574	1:25
pos HSE	0,009	0,427	1:25
Pos MIN	0,01	0,513	1:25

Table 2: UV-VIS Absorption and calculated DNA concentration together with the dilution factor applied therefore for PCR for each sample. The samples are denominated as follows: “I” is for inducible lines transformed by pBI-HSE-35MinGUS, “G” for constitutively expressing GUS lines not used any further, “M” for minimal promotor GUS transformed by pBI-35MinGUS, “pos HSE” and “pos MIN” are positive controls of pBI-HSE-35MinGUS and pBI-35MinGUS isolated from corresponding transgenic *E. coli*.

PCR

Samples from the finished PCR were put to a 0.8% agarose gel to check for PCR products indicating a DNA insert and therefore positive transformation of *Arabidopsis* plants.

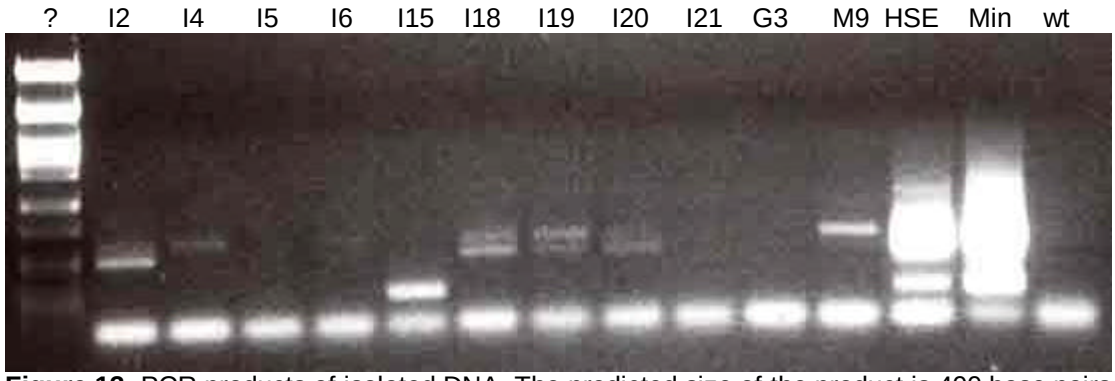

Figure 13: PCR products of isolated DNA. The predicted size of the product is 400 base pairs (bp), which is visible for I2, I4, I6 (very weak), I18, I19, I20, M9 and the two positive controls HSE (posHSE from DNA isolation) and Min (posMin from DNA isolation). A second band appears at 600 bp for unknown reasons. Denomination of lines see comments on **table 1.**

As can be seen in **figure 13** the following lines can be considered positive transformants as at least a visible band appeared on the gel, indicating the presence of a PCR product: I2, I18 and I19 to be used for phenotype analysis. The lines I4, I6 and I20 were also positive (although in line I6 GUS-staining was barely visible), but were not used because of their weak response in GUS stainings. The reason for the second band showing at 600 bp and appearing above the predicted PCR product of 400 bp is unknown; primer mismatches were excluded by sequence comparison.

5.3. Segregation analysis

To estimate the copy number of gene inserts in transformed *Arabidopsis* plants, second generation seeds were analyzed for their antibiotic resistance (acquired through transformation), where the rate of living seedlings to dead ones (following the classical Mendelian rules) resulted in the copy number of the transformed T-DNA.

Transgenic Line	Total Plants	Plants Survived	Plants Died	Ratio
I2	153	99	54	3:1
I4	94	70	24	3:1
I6	16	13	3	3:1
I18	37	27	10	3:1
I19	370	303	67	3:1
I20	268	242	26	>3:1
I21	44	41	3	>3:1

Table 3: Plant number identifies the individual plant and its transgenic background, where "I" means inducible, containing pBI-HSE-35Min-GUS.

A ratio alive to dead (transgenic to wildtype) of about 3:1 suggests only one copy of transgene in the originally transformed plants and therefore also in the T2 generation used for fluorometric GUS assay. A higher ratio (>3:1) suggests two or more independent copies of the transgene in the plants. First generation seeds were still subject to GUS histochemical staining to have several criteria to choose plants for fluorometric GUS assay.

5.4. GUS Histochemical staining

The transformed plants of the first generation were used for GUS histochemical staining to check again for positive transformants, for functionality of the HSE insert and to choose plants for quantitative fluorometric GUS assay.

The following summary shows the different plants staining results.

Plant Nr.	Temp. (°C)	Leaf	Root	Stem	Inflourescence	Plant Nr.	Temp. (°C)	Leaf	Root	Stem	Inflourescence
I1	22	+	+			I10	4	-	+	+	-
	33	+	++				17	+	-	+	+
	37	+	+++				22	+	-	+	-
	42	+	++				33	+	+	+	-
I2	22	+	+				37	+	++	++	-
	33	++	++				42	na	na	na	Na
	37	++	++			I11	4	+	-	-	-
	42	+	+				17	-	-	+	-
I3	22	+	-				22	+	-	++	-
	33	+	+				33	++	+	+++	-
	37	+	+				37	+++	++	+++	-
	42	+	-				42	-	-	+	-
I4	22	+	-			I12	4	++	+	+	+
	33	+	+				17	-	-	++	-
	37	+	++				22	+	+	++	-
	42	+	+				33	++	+++	++	-
I5	4	-	-	-	-		37	+++	+++	+++	+
	17	-	-	-	-		42	++	-	++	-
	22	-	-	-	-	I13	4	+-	+-	+	-
	33	-	-	-	-		17	++	+-	+	Na
	37	-	-	-	-		22	++	+-	+-	Na
	42	-	-	-	-		25	+-	+-	-	Na
I6	4	+	+	+	-		33	+++	+-	+	Na
	17	+	+	+	+		37	++++	++	+	Na
	22	+	+	++	+		42	+++	+	+	Na
	33	+	+	++	na	I14	4	+	+	+	Na
	37	+	-	++	-		17	+	+	+	Na
	42	+	+	+	-		22	-	+	-	Na
I7	4	+	+	+	-		25	-	+	+	Na
	17	+	+	+	+		33	-	+	+	Na
	22	+	+	+	+		37	+	+	+	Na
	33	+	+	+++	+		42	+	-	-	Na
	37	+	-	+	+	I15	4	-	-	+	Na
	42	+	-	+	-		17	-	+-	+	Na
I8	4	+	+	+	na		22	+	+	-	Na
	17	+	-	-	-		25	+	+	-	Na
	22	+	-	+	na		33	++	-	++	Na
	37	++	+	++	+		37	+++	+	+	Na
	42	-	-	-	-		42	+	+-	+++	Na
I9	4	+	-	-	+						
	17	++	-	na	-						
	22	-	-	+	-						
	33	-	-	++	-						
	37	+	-	++	-						
	42	+	-	+	-						

Table 4: Staining results summary of first screen. Explanation for stainings: "-" is no visible staining, "+-" unclear staining, "+" weak visible staining, "++" clearly visible staining and "+++" strong visible staining.

Some promising plants were screened more closely in a serial experiment.

Staining Nr.		1				2				3			
Plant Nr.	Temp (°C)	Leaf	Root	Stem	Inflourescence	Leaf	Root	Stem	Inflourescence	Leaf	Root	Stem	Inflourescence
M11	4	-	-	-	-	-	-	-	-	-	+-	-	-
	17	-	-	-	-	-	-	-	-	-	-	-	-
	25	-	-	-	-	-	-	-	-	-	-	-	-
	33	-	-	-	-	-	-	-	-	-	-	-	-
	37	-	-	-	-	-	-	-	-	-	-	-	-
	42	-	-	-	-	-	-	-	-	-	-	-	-
I18	4	+	-	+	+	+	-	+	-	-	++	-	-
	17	+	-	+	-	+	-	++	-	-	++	-	-
	25	+	+	+	++	+	++	+	++	-	+	+	-
	33	+	++	+++	++	++	+	+	+	-	+++	+	-
	37	+++	+++	+++	+++	+	+++	+++	+++	+	+++	+++	+++
	42	++	+	+++	na	+++	++	+++	++	+	++	+	-
19	4	-	-	+	-	-	++	++	-	-	-	+	-
	17	-	+	+	+	+-	-	+-	-	-	-	+	-
	25	-	-	+	+	+-	+-	+	-	-	+	++	+
	33	+	++	++	na	+	+	++	++	-	+	-	+
	37	++	++	+++	++	++	++	+++	+++	++	-	++	++
	42	++	na	++	-	-	+	++	-	-	+	++	++
I20	4	-	-	+-	-	-	-	-	-	-	+-	-	++
	17	+-	+-	+-	-	-	-	-	-	-	-	-	-
	25	-	+-	-	-	-	+-	+	-	-	-	-	+
	33	+-	-	+	-	+	-	++	-	-	-	+	+
	37	+	+	+++	+++	+	++	++	na	-	-	+++	++
	42	-	+	+	-	-	+	++	-	-	-	+	-
I21	4	-	-	-	-	-	-	+++	-	-	-	+-	-
	17	-	-	++	-	-	-	++	-	-	-	+	-
	25	-	-	+	-	-	-	+++	-	-	-	+-	-
	33	++	+	++	+	-	-	++	-	-	-	+	-
	37	+++	+	+++	+	++	+	++	++	++	+	++	+
	42	+	-	+	+	+	-	+	-	-	-	+	-

Table 5: Staining results summary of serial screens done with some promising lines. Explanation for stainings: "-" is no visible staining, "+-" unclear staining, "+" weak visible staining, "++" clearly visible staining and "+++" strong visible staining.

After careful considerations, plants I2, I18 and I19 were selected (together with wildtype plants as control) using leave and stem tissue for quantitative fluorometric GUS assay. The results leading to this decision are highlighted in tables 4 and 5. These plants showed almost no GUS activity when exposed to temperatures from 4 °C to 25 °C and very high GUS activity at 37°C.

5.5. Fluorometric GUS Assay

Standard series

Along with every measurement series a dilution row of 4-methyl-umbelliferone was included as standards with defined concentrations.

Standard concentration	Fluorescence Intensity of Standards series 1	Fluorescence Intensity of Standards series 2	Fluorescence Intensity of Standards series 3
1,E-13	2980	3392	2412
1,E-11	1176	2121	2029
1,E-09	5064	7209	6810
1,E-07	8371	6270	6926
1,E-05	467792	351911	337290
1,E-03	8342368	8055720	5708280

Table 6: The shaded standards appear to be in the linear range, whereas the other, non-shaded standards do not obey the law of Lambert-Beer (Linear correlation between fluorescence intensity and concentration of fluorescent substance, e.g. 4-MU) and where not utilized in further calculations.

From these standards the slope and the intercept of the regression line (following the simple equation for linear correlations y = k * x + d, where k is the slope and d the intercept in a xy coordinate system) were calculated.

Standard concentration	Fluorescence Intensity of Standards series 1	Fluorescence Intensity of Standards series 2	Fluorescence Intensity of Standards series 3
1,E-11	1176	2121	2029
1,E-09	5064	7209	6810
1,E-07	8371	6270	6926
slope	53064203064,20	16519422769,42	25545156795,16
Intercept	3083,66	4643,67	4394,89

Table 7: Slope and intercept of the y values fluorescence intensity and the x values standard concentration.

To calculate the concentration of 4-MU the values from the raw data (umbelliferone counts, see **table 8**) were used, following the equation y = k * x + d, where y is the fluorescence intensity and x the concentration of 4-MU, k the slope and d the intercept of the y axis, both determined from the standards. Parallel to the fluorescence measurement the protein concentration of the used extracts was

determined by Bradford protein assay, which was used to calculate the GUS activity (nmol of 4-MU produced per minute per mg protein) for an incubation time of 24 hours. All these values are listed in the following table (**Table 8**), sorted by the number of series of measurement.

Series		1				2				3			
Plant Nr.	Temp (°C)	Umb Counts	4-MU conc (nmol)	Prot (mg/ml)	GUS act	Umb Counts	4-MU conc (nmol)	Prot (mg/ml)	GUS act	Umb Counts	4-MU conc (nmol)	Prot (mg/ml)	GUS act
I2l	**4**	37525	649	0,394	**1,14**	14090	572	0,594	**0,67**	15758	445	0,457	**0,68**
	17	20535	329	0,544	**0,42**	15667	667	0,588	**0,79**	20827	643	0,513	**0,87**
	25	21360	344	0,219	**1,09**	45628	2481	0,725	**2,38**	25895	842	0,551	**1,06**
	33	31174	529	0,250	**1,47**	42553	2295	0,544	**2,93**	42090	1476	0,307	**3,34**
	37	199352	3699	0,263	**9,77**	42353	2283	0,769	**2,06**	289564	11163	0,476	**16,29**
	42	19604	311	0,150	**1,44**	55867	3101	0,657	**3,28**	12937	334	0,469	**0,50**
I2S	**4**	14867	222	0,476	**0,32**	11340	405	0,432	**0,65**	6589	86	0,407	**0,15**
	17	18561	292	0,006	**33,76**	14290	584	0,063	**6,44**	8275	152	0,876	**0,12**
	25	21011	338	0,043	**5,46**	13004	506	0,194	**1,81**	9756	210	0,645	**0,23**
	33	65320	1173	0,031	**26,27**	30612	1572	0,225	**4,85**	67208	2459	0,695	**2,46**
	37	98083	1790	0,169	**7,36**	171309	10089	0,369	**18,99**	155166	5902	1,064	**3,85**
	42	9449	120	0,000		214753	12719	0,213	**41,47**	11935	295	0,482	**0,43**
I18L	**4**	15553	235	0,250	**0,65**	27399	1377	0,113	**8,47**	12036	299	0,388	**0,54**
	17	19218	304	0,038	**5,56**	13378	529	0,188	**1,95**	81829	3031	0,463	**4,55**
	25	16439	252	0,113	**1,55**	15862	679	0,494	**0,95**	12704	325	0,451	**0,50**
	33	52965	940	0,000		17370	770	0,300	**1,78**	24251	777	0,451	**1,20**
	37	72708	1312	0,000		200558	11860	0,125	**65,89**	387369	14992	0,482	**21,60**
	42	11253	154	0,106	**1,01**	25725	1276	0,094	**9,43**	7042	104	0,551	**0,13**
I18S	**4**	11895	166	0,188	**0,61**	8209	216	0,307	**0,49**	64916	2369	0,901	**1,83**
	17	9047	112	0,131	**0,60**	8403	228	0,194	**0,81**	8059	143	0,707	**0,14**
	25	9476	120	0,000		11617	422	0,144	**2,04**	9641	205	0,432	**0,33**
	33	9100	113	0,219	**0,36**	15248	642	0,113	**3,95**	52669	1890	0,682	**1,92**
	37	170878	3162	0,206	**10,66**	145091	8502	0,113	**52,25**	118906	4483	0,300	**10,38**
	42	11615	161	0,206	**0,54**	81420	4648	0,106	**30,45**	4687	11	0,757	**0,01**
I19L	**4**	26959	450	0,557	**0,56**	42805	2310	0,544	**2,95**	13903	372	0,732	**0,35**
	17	31897	543	0,338	**1,12**	14224	580	0,263	**1,53**	13298	349	0,526	**0,46**
	25	64757	1162	0,332	**2,43**	10673	365	0,212	**1,20**	70913	2604	1,089	**1,66**
	33	79760	1445	0,369	**2,72**	35141	1846	0,019	**67,48**	18725	561	0,482	**0,81**
	37	559076	10478	0,407	**17,88**	196020	11585	0,194	**41,47**	53790	1934	0,619	**2,17**
	42	17725	276	0,307	**0,62**	55157	3058	0,106	**20,03**	6320	75	0,469	**0,11**
I19S	**4**	10163	133	0,275	**0,34**	7927	199	0,056	**2,47**	6435	80	0,663	**0,08**
	17	19176	303	0,269	**0,78**	13320	525	0,232	**1,57**	153138	5823	0,563	**7,18**
	25	7771	88	0,004	**15,34**	90344	5188	0,181	**19,90**	9773	211	0,482	**0,30**
	33	84588	1536	0,213	**5,01**	33890	1770	0,344	**3,57**	72985	2685	0,526	**3,54**
	37	103418	1891	0,181	**7,25**	89741	5151	0,006	**596,2**	159314	6065	0,820	**5,14**
	42	5662	49	0,138	**0,24**	234214	13897	0,004	**2244**	5642	49	0,244	**0,14**
WTL	**4**	-611	-70	1,821	**-0,03**	2190	-149	1,352	**-0,08**	-924	-208	2,490	**-0,06**
	17	880	-42	1,840	**-0,02**	2088	-155	1,333	**-0,08**	4415	1	1,683	**0,00**
	25	9169	115	1,589	**0,05**	8420	229	0,914	**0,17**	-1484	-230	2,027	**-0,08**
	33	5192	40	1,076	**0,03**	4262	-23	1,189	**-0,01**	2554	-72	1,896	**-0,03**
	37	6816	70	0,738	**0,07**	5033	24	0,870	**0,02**	3086	-51	1,871	**-0,02**
	42	3143	1	0,807	**0,00**	5678	63	0,519	**0,08**	3014	-54	1,107	**-0,03**

WTS	**4**	5004	36	0,832	**0,03**	2256	-145	0,901	**-0,11**	1443	-116	1,320	**-0,06**
	17	3648	11	0,594	**0,01**	4653	1	0,588	**0,00**	318	-160	1,927	**-0,06**
	25	4181	21	0,857	**0,02**	4538	-6	0,019	**-0,23**	1693	-106	1,965	**-0,04**
	33	6689	68	0,907	**0,05**	4231	-25	0,451	**-0,04**	880	-138	1,965	**-0,05**
	37	5026	37	0,751	**0,03**	1339	-200	1,145	**-0,12**	3766	-25	1,283	**-0,01**
	42	5073	37	0,763	**0,03**	3626	-62	0,131	**-0,33**	2649	-68	0,551	**-0,09**

Table 8: Summary of fluorescence intensity (Umb red by blanc), 4-MU concentration (nmol/l), protein concentration of the sample (mg/ml) and the activity of GUS (nmol/min/mg protein) of all series of measurement.

From each series of measurement an average was calculated per sample per temperature. Because of high standard deviation certain values were not included in the average value and considered as measurement errors. Those values are marked red in the following table (**Table**). The averages were then used for an illustrative diagram.

Series		**1**	**2**	**3**	Average GUS activity	Standard Deviation
Plant Nr.	**Temp (°C)**	**GUS act**	**GUS act**	**GUS act**		
I2I	**4**	1,14	0,67	0,68	**0,83**	0,22
	17	0,42	0,79	0,87	**0,69**	0,20
	25	1,09	2,38	1,06	**1,51**	0,61
	33	1,47	2,93	3,34	**2,58**	0,80
	37	9,77	2,06	16,29	**9,37**	5,81
	42	1,44	3,28	0,50	**1,74**	1,16
I2S	**4**	0,32	0,65	0,15	**0,37**	0,21
	17	33,76	6,44	0,12	**3,28**	**14,60**
	25	5,46	1,81	0,23	**2,50**	2,19
	33	26,27	4,85	2,46	**3,65**	**10,71**
	37	7,36	18,99	3,85	**10,07**	6,47
	42		41,47	0,43		**20,52**
I18L	**4**	0,65	8,47	0,54	**3,22**	3,71
	17	5,56	1,95	4,55	**4,02**	1,52
	25	1,55	0,95	0,50	**1,00**	0,43
	33		1,78	1,20	**1,49**	0,29
	37		65,89	21,60	**21,60**	22,14
	42	1,01	9,43	0,13	**3,52**	4,19
I18S	**4**	0,61	0,49	1,83	**0,98**	0,60
	17	0,60	0,81	0,14	**0,52**	0,28
	25		2,04	0,33	**1,18**	0,85
	33	0,36	3,95	1,92	**2,08**	1,47
	37	10,66	52,25	10,38	**21,04**	**19,67**
	42	0,54	30,45	0,01	**0,55**	**14,22**

I19L	**4**	0,56	2,95	0,35	**1,29**	1,18
	17	1,12	1,53	0,46	**1,04**	0,44
	25	2,43	1,20	1,66	**1,76**	0,51
	33	2,72	67,48	0,81	**3,53**	**30,99**
	37	17,88	41,47	2,17	**20,51**	16,15
	42	0,62	20,03	0,11	**6,92**	9,27
I19S	**4**	0,34	2,47	0,08	**0,96**	1,07
	17	0,78	1,57	7,18	**3,18**	2,85
	25	15,34	19,90	0,30	**11,85**	**8,37**
	33	5,01	3,57	3,54	**4,04**	0,68
	37	7,25	596,2	5,14	**6,20**	**278,14**
	42	0,24	2244	0,14	**0,38**	**1057,90**
WTL	**4**	-0,03	-0,08	-0,06	**-0,05**	0,02
	17	-0,02	-0,08	0,00	**-0,03**	0,03
	25	0,05	0,17	-0,08	**0,05**	0,10
	33	0,03	-0,01	-0,03	**0,00**	0,02
	37	0,07	0,02	-0,02	**0,02**	0,03
	42	0,00	0,08	-0,03	**0,02**	0,05
WTS	**4**	0,03	-0,11	-0,06	**-0,05**	0,06
	17	0,01	0,00	-0,06	**-0,01**	0,03
	25	0,02	-0,23	-0,04	**-0,08**	0,11
	33	0,05	-0,04	-0,05	**-0,01**	0,05
	37	0,03	-0,12	-0,01	**-0,03**	0,06
	42	0,03	-0,33	-0,09	**-0,13**	0,15

Table 9: Average GUS activity of all experiments, together with outliers not included in the averages (marked in red) and the standard deviation for each set of values.

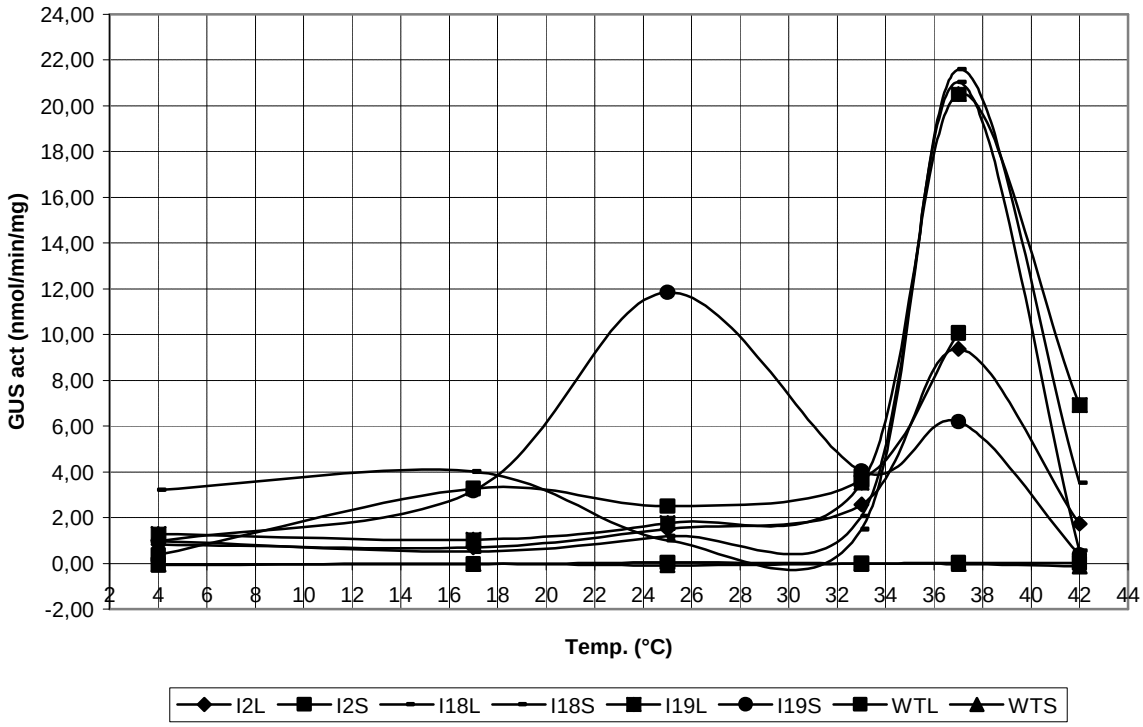

Diagram 1: Summary of all lines (including Wildtype, barely visible along the x axis), showing clearly the inducible properties of the heat shock enhancer, but also revealing background expression.

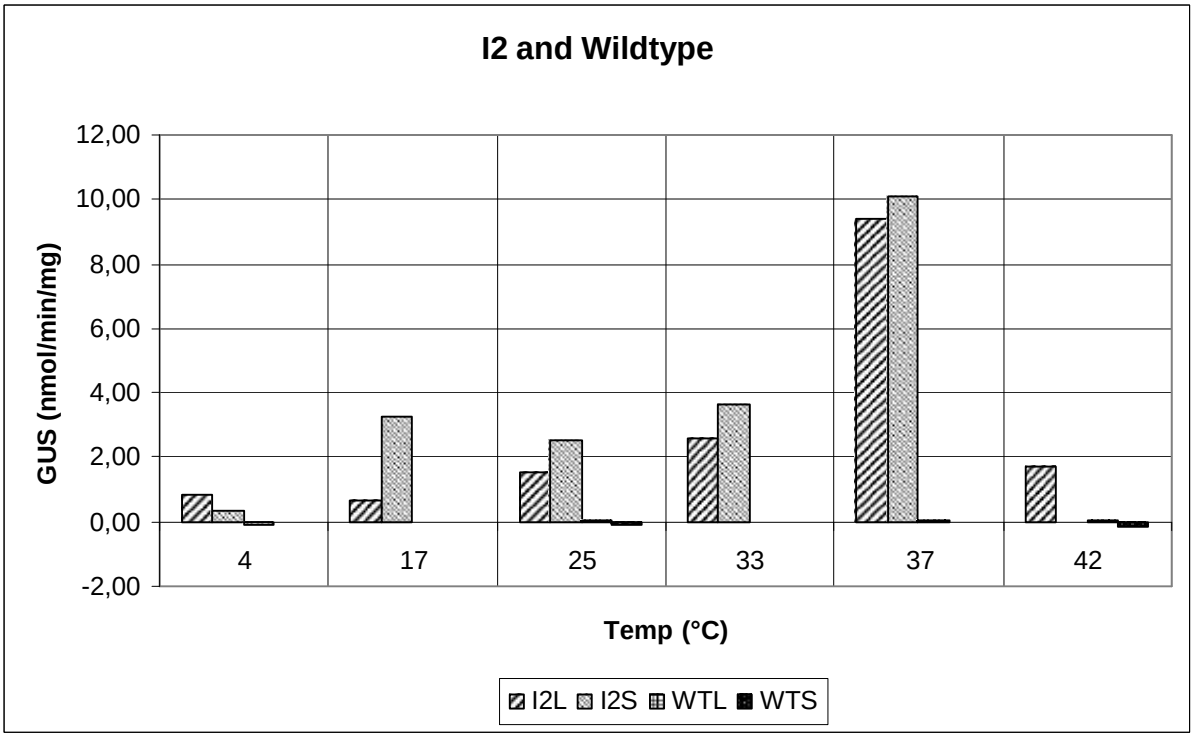

Diagram 2: Temperature dependent GUS activity in the inducible line I2, showing the temperature dependent GUS expression of the HSE element.

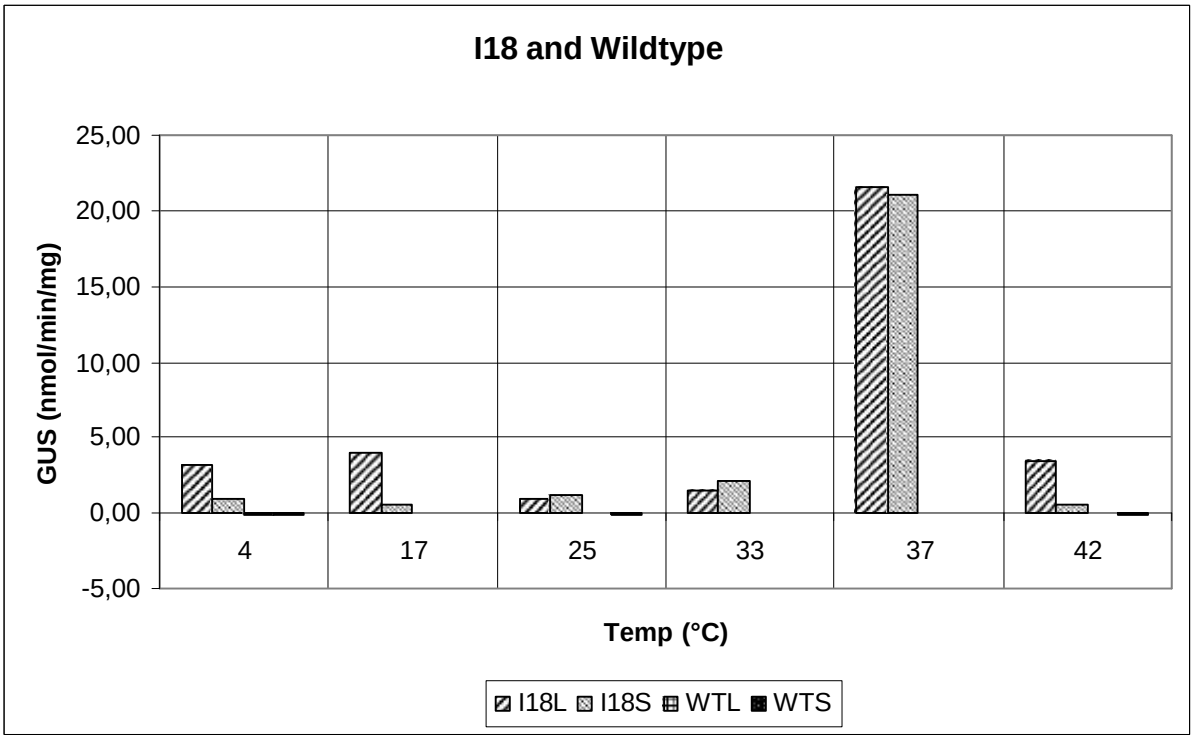

Diagram 3: Temperature dependent GUS activity in the inducible line I18, showing inducible behaviour as expected with a maximum at 37°C and rather low to zero activity at 25°C and low activity at 17°C and 4°C.

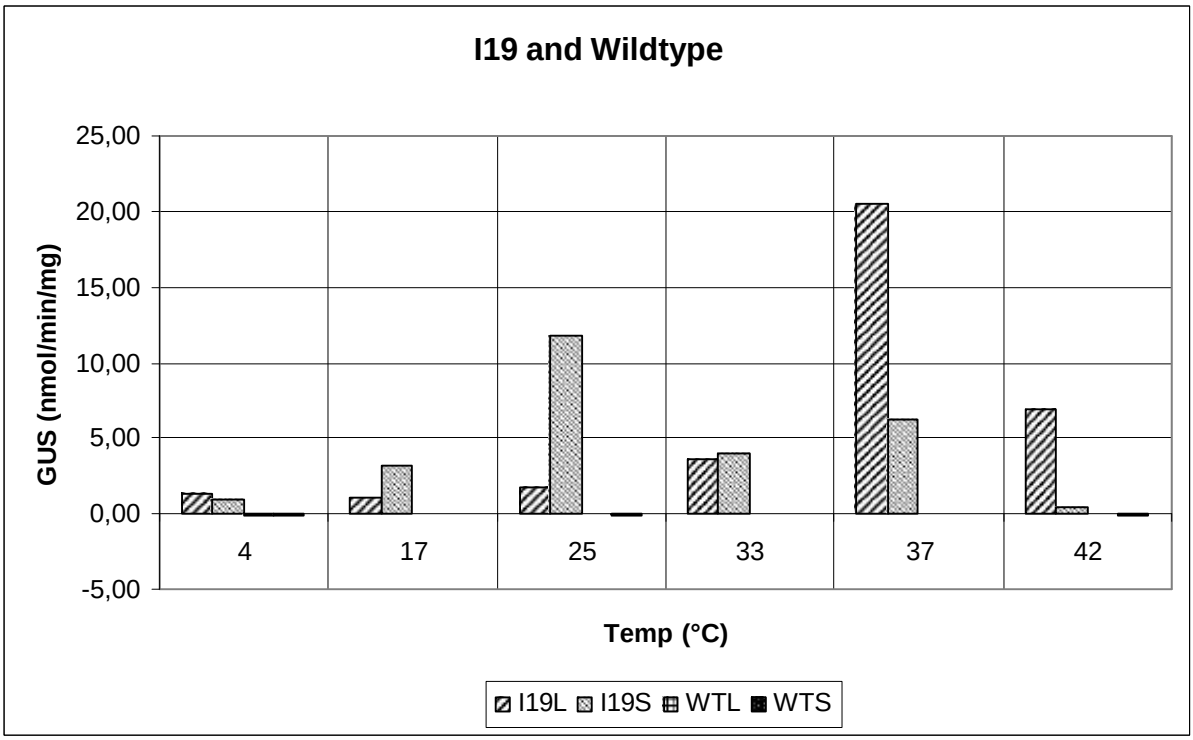

Diagram 4: Temperature-dependent GUS activity in the inducible line I19. Also here a measurement error seems obvious at 25°C for I19S, where the standard deviation is moderately high (8,37).

As one can see in the diagrams (especially **diagram 2** to **4**), all lines transformed by pBI-HSE-35MinGUS show increased GUS activity at 37°C and low GUS activity at 25°C (with exceptions marked in the text below each diagram). During the experiments it became obvious that explants were severely damaged by treatment at 42°C, resulting on the one hand in a strong decrease in GUS activity, on the other hand a great variance in protein concentration in the samples resulting in a greater variance in GUS activity values. This may explain the in some lines visible increase in GUS activity, as it is calculated per mg protein.
Quite interesting is the fact of moderately increasing GUS activity at temperatures below 25°C.

6. DISCUSSION

So far, existing inducible genetic systems fulfil the requirements expected from them (Gatz, 1997) only partially:

- Without induction the expression of the controlled gene should be the least leaky possible.
- Induction should be dose-dependent and induction levels should span over at least three orders of magnitude.
- The expression pattern should be homogenous in all tissue of the organism.
- Inducing conditions should bare minimal consequences on the organism's physiology, fitness and viability.

Especially the last two requirements are an issue for chemically inducible systems, depending of the chemical nature of the inducer. Uptake, transport and distribution between plant tissues can be considerable barriers to chemical inducers, preventing uniform expression of the transgene, reducing induction efficiency and delaying the time of induced expression. Some inducers (most obviously ethanol) also show toxic effects when applied to the transformed plant with concentrations close to the optimum for induction of the transgene, severely influencing plant physiology and viability. The chemically inducible systems presented in chapter 1.2.1. are therefore not ideal following the principals suggested by Christine Gatz, though having so far

contributed in a large quantity of very interesting studies and being successfully applied to a similar number of plant model systems.

To achieve a more widespread and uniform induction within a transgenic plant an environmental cue would be more suitable then a chemical one. The application of heat as an inducing cue seems more appropriate simply because plants are quite adapted to cope with increasing temperature. Induction via the small heat shock proteins (shsp's) was also mentioned earlier in this work and presents a quite suitable system, using expression cassettes driven by heat-regulated promoters (Ainley and Key, 1990; Schöffl et al., 1989). An alternative to the application of these cassettes is the use of enhancer elements, being only a very small (about 150 bp) piece of DNA that can be easily integrated into any existing expression system including a minimal promoter in front of the transgene and/or reporter gene, conferring gene expression to otherwise silent genes. Heat sensitive enhancer systems are therefore a logical step towards improved inducible systems meeting the requirements listed at the beginning of the chapter. An artificial enhancer consisting of a multimer of ideal binding sites for heat shock factors would be a good candidate for this purpose. When introduced into Medaka fish, this enhancer showed superior properties in comparison to natural heat shock promoters because of its artificial structure: dramatically reduced background activity, improved inducibility and loss of all tissue specific components (Bajoghli et al., 2004).

When introduced into *Arabidopsis thaliana*, the results were similar to Medaka fish, showing an increase of GUS expression from at 37°C which was 13 times higher than at 25°C. However, the increase of GUS activity was not linear but exponentially rising over 33°C to 37°C. This is similar to heat shock induced GUS expression in *Physcomitrella patens* using a hsp17.3B promotor of a soybean class I sHSP (Saidi et al., 2005). At lower temperatures (4°C and 17°C) there was not a clear increase in GUS expression in the quantitative assay hinting to a similar response to cold stress as to heat stress in *Arabidopsis*. However, results from GUS stainings indicate that such a cold response might exist. Heat shock response in plants is also activated by other stimuli such as treatment with acetyl salicylic acid and benzyl alcohol (Saidi et al.,2005). Background expression at 25°C was also low, with exceptions mentioned in chapter 5.5 where one line showed (I19S) showed abnormally high GUS expression at 25°C, but also a high standard deviation (of **11,85**), so can be seen as .

So, induction of GUS activity was independent of the tissue used for the experiment, proving the uniformity of induction granted by the artificial heat shock enhancer.
Referring to the requirements proposed by Christine Gatz, the heat shock enhancer element presents itself as a quite interesting and useful one, though it does not meet al requirements completely:

- *Without induction the expression of the controlled gene should be the least leaky possible.*

With the exception of the sample from plant I19 stem tissue all other samples showed low GUS activity levels close to zero when no heat shock or cold stress was applied. However, when exposed to low temperatures, GUS activity was in some cases slightly higher than at 25°C. This was also visible in the histochemical GUS stainings, probably indicating a similar response to cold stress as to heat stress. Since the used enhancer element consists exclusively of multimerized binding sites to heat shock genes, the same proteins are also activated when plants are exposed to cold stress.

- *Induction should be dose-dependent and induction levels should span over at least three orders of magnitude.*

The total span of induction is not greater than one order of magnitude, so this requirement is not completely met.

- *The expression pattern should be homogenous in all tissue of the organism.*

When comparing the GUS activity in tissues of the same plants, no clear difference can be seen: in plant I2 GUS activity in leaves is 9,37 nmol/mg/sec and in stem tissue it is 10,07 nmol/mg/sec at 37°C, so the values are almost equal. In the other plants tested, the values of GUS activity are not so homogenous (I18 leaf 21,60 nmol/mg/sec and stem 21,04 nmol/mg/sec, I19 leaf 20,51 nmol/mg/sec and stem 6,20 nmol/mg/sec, it has to be noted that this whole series shows deviating behaviour and its measurement values should be questioned as a whole), the expression of GUS is also close to equal in the two measured tissues, giving strong support to the properties claimed by Bajoghli et al. (2004).

So, induction of GUS activity was independent of the tissue used for the experiment, proving the uniformity of induction granted by the artificial heat shock enhancer.

- *Inducing conditions should bare minimal consequences on the organism's physiology, fitness and viability.*

When applying heat to the sample explants, no effect was visible up to 37°C, where the highest induction of GUS activity was achieved. Only at 42°C the sample explants were effected, showing brownish spots on the tissues, especially leaves. Since GUS was not strongly induced at that temperature, this requirement can also be counted as fulfilled.

7. SUMMARY

Overall, the HSE system first introduced in Medaka fish (Bajoghli et al., 2004) also works well in *Arabidopsis thaliana*, adding a very versatile inducible system to the repertoire of this model system. Because of its simplified nature, an adaption to other plant model systems seems quite likely and should prove not a great barrier, allowing the study of plant genes under very tight control to better understand their function.
As could be shown, the requirements proposed by Christine Gatz for an ideal inducible genetic system all but one are met:

- Without induction, GUS activity is near zero, though slightly higher then in wildtype plants (in average **1,59** nmol/mg/min)
- Induction of GUS is independent of the tissue, with an average difference between the two tissues measured (leaf and stem) of **0,61** nmol/mg/min.
- The conditions for induction had no negative impact on the explants, only a heat treatment of 42°C left the explants damaged (as expected).
- The only condition that induction should at least span three orders of magnitude was not met; the reason for this could be simply a different optimal temperature for induction than used in these experiments and has yet to be determined.

7. Literature

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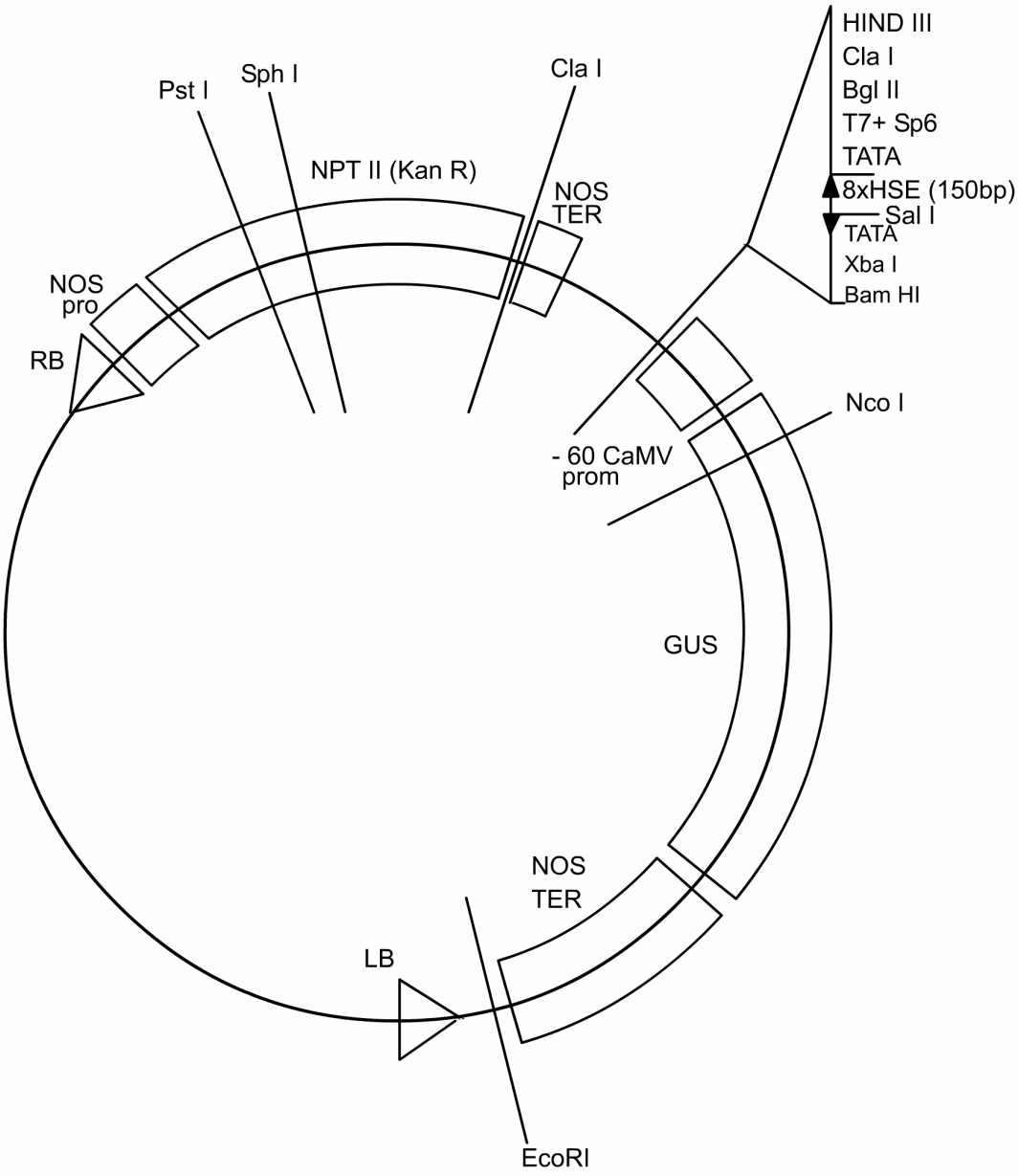
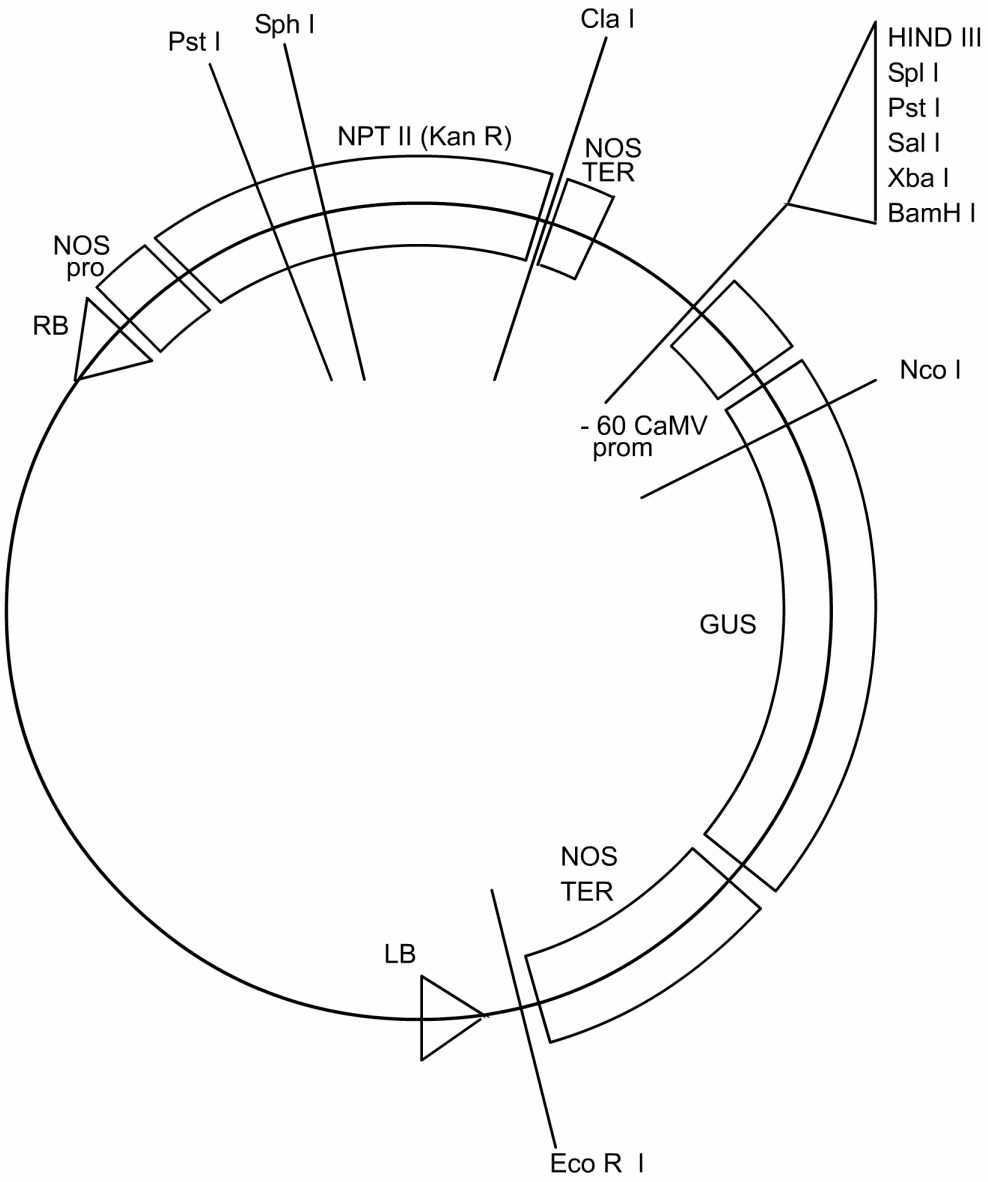
Appendix: Plasmid Constructs

Plasmid name: pBI-HSE-35Min-GUS
Vector: pBI-35Min-GUS
Insert: 8x HSE from pSGH2
Resistance Markers: KanR
Creator: Martin Kragl
Cloning Site: HindIII/SalI



Plasmid name: pBI-35Min-GUS
Vector: pBI101.1
Insert:CaMV-GUS from p221.9GRE6
Resistance Markers:KanR
Creator: Martin Kragl
Cloning Site: BamHI/EcoRI



ZUSAMMENFASSUNG

Das HSE System, zuerst getestet in Medaka (Bajoghli et al., 2004) funktioniert soweit gut in *Arabidopsis thaliana*, und fügt dem Repertoire dieses Modellsystems ein sehr vielseitiges induzierbares System hinzu. Durch den stark vereinfachten Aufbau kann man davon ausgehen, dass es auch in anderen Modellsystemen für Pflanzen Anwendung finden wird, was keine größeren Schwierigkeiten darstellen sollte, um die Funktion von Pflanzengenen unter strikter Kontrolle zu ermöglichen.
Durch die Ergebnisse dieser Arbeit konnte bewiesen werden, dass fast alle Kriterien, die von Christine Gatz für ein ideales induzierbares genetisches System erfüllt werden:

? Ohne induzierende Bedingungen ging die Aktivität von GUS gegen Null (**1,59** nmol/mg/min) im Durschnitt , obwohl sie noch immer knapp über der Aktivität der Wildtyp Pflanzen lag.

? Weiters konnten keine Unterschiede in den induzierten GUS Aktivitäten zwischen den beiden verwendeten Pflanzengeweben (Blatt und Stiel) festgestellt werden (Durchschnittlicher unterschied zwischen den beiden Geweben: **0,61** nmol/mg/min).

? Die induzierenden Bedingungen verursachten keinerlei Schäden bei den Pflanzenteilen, erst bei 42°C wurden diese wie erwartet in Mitleidenschaft gezogen.

? Das einzige Kriterium, das nicht voll erfüllt wurde, war die, dass die Aktivität des Reportergens durch die Induktion um mindestens drei Größenordnungen gesteigert werden sollte. Dies kann an einer anderen optimalen Aktivierungstemperatur liegen, die erst noch zu bestimmen ist.

Ein weiteres Ergebnis, die Induktion von GUS durch das HSE Element durch niedrigere Temperaturen konnte nur in histochemischen Färbungen beobachtet werden. In quantitativen fluorometrischen Bestimmungen konnte dies nicht wiederholt bzw. bestätigt werden. Eine möglicherweise vergleichbare Reaktion auf Kältestress bzw. Hitzestress kann also noch nicht bestätigt werden, sollte jedoch aufgrund der verschiedenartigen Ergebnisse dieser Arbeit noch genauer untersucht werden.

Englischspachige Zusammenfassung: siehe Seite 55

Europass Curriculum Vitae

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Work experience

Dates	From February 2008 to September 2009
Occupation or position held	**Sales Assistant**
Main activities and responsibilities	Sales, customer support, event management
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Type of business or sector	Games and Hobby
Dates	August 2003
Occupation or position held	**Technical Assistant/ Trainee**
Main activities and responsibilities	Associate project :'Radiation induced decomposition of polyaromatic combustion products
Name and address of employer	Institute for radiation chemistry, University of Vienna
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Type of business or sector	Pharma industry
Dates	August 1999, 2000 and 2001

Occupation or position held	**Archivist**
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Education and training

Dates	October 1999 to February 2010
Title of qualification awarded	Master, exam 01.03.2010
Principal subjects/occupational skills covered	Basics in Botany, Zoology, Ecology, Paleontology, Advanced Microbiology, Genetics, molecular biology, developmental biology, biochemistry
Name and type of organisation providing education and training	**University of Vienna**, Department for Life Sciences
Level in national or international classification	University
Dates	October 1998 to May 1999
Title of qualification awarded	
Principal subjects/occupational skills covered	**Military service**
Name and type of organisation providing education and training	"Österreichisches Bundesheer", Casern Hörsching
Level in national or international classification	
Dates	September 1993 to June 1998
Title of qualification awarded	
Principal subjects/occupational skills covered	Anorganic and organic chemistry, analytical and preparative chemistry, engineering basics, electronic basics
Name and type of organisation providing education and training	**HTBLA Wels**, Department for technical chemistry
Level in national or international classification	High School

Personal skills and competences

Mother tongue(s) **German**

Other language(s)

Self-assessment	**Understanding**				**Speaking**				**Writi**	
European level (*)	Listening		Reading		Spoken interaction		Spoken production			
English		Advanced		Advanced		Advanced		Advanced		Adva
Spanish		Beginner		Beginner		Beginner		Beginner		Beg

	(*) Common European Framework of Reference for Languages
Social skills and competences	Team oriented, accurate, responsible, curious , patient, tolerant, customer- oriented,humorous, talent improvise
Organisational skills and competences	✍ Event organisation (Games Workshop, Gamesday Köln 2008 and Spielefest Vienna 2008), ✍ storage management (Salvagnini, Novo Nordisk, Games Workshop) ✍ Projektmanagement of Master tesis University Vienna ✍ Tutor in "Übungen in Cyto- und Entwicklungsgenetik" 2007
Technical skills and competences	✍ molecular biology (PCR, transgene cloning, various transformation techniques, RNA isolati RT-PCR, Protein isolation and western blot,), ✍ cell cultures (plant cells and explants), ✍ analytical techniques (various chromatography techniques, qualitative analysis, UV-VIS Spectroscopy, Atomic absorption spectroscopy, mass spectrometry, technical analysis of oi steel, cement, fertilizer, et.), ✍ preparative organic chemistry (synthesis and purification)
Computer skills and competences	Advanced Windows system software and office (EBCL niveau), Basic hardware and UNIX (Linux)
Artistic skills and competences	Miniature constructing and painting (Hobby)
Driving licence	Type B
Publications list	Fuga L, Kragl M, Getoff N (2004) Vitamin B2 (riboflavin) and a mixture of vitamin B2 and C affects MI efficiency in aerated media under irradiation. Anticancer Res. 24(6):4031-4
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