Study of genes modifying morphology, pathogen interactions and MEP-derived metabolites during barley root colonization by *Piriformospora indica* via stable root transformation system

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Contents

List of abbreviations

BCID	Barley callus induction medium
Bgh	Blumeria graminis f.sp. hordei
BI-1	Bax inhibitor-1
BLAST	Basic local alignment search tool
bp	Base pair
cDNA	Complementary DNA
CoA	Acetyl co-enzyme A
CPS	Ent-copalyl diphosphate synthase
cv.	Cultivar
Da	Dalton
Dai	Day after inoculation
DEPC	Diethylpolycarbonate
DMAPP	Dimethylallyl diphosphate
DTT	DL-Dithiothreitol
DNA	2'-desoxy-ribonucleic acid
dsRNA	Double stranded RNA
E. coli	Escherichia coli
ER	Endoplasmic Reticulum
EST	Expressed sequence tag
EV	Empty vector

et.al	Et altera
FHB	Fusarium head blight
GA	Gibberellic acid
GFP	Green fluorescent protein
GGDP	Geranylgeranayl diphosphate
GP	Golden Promise
GUS	β-glucuronidase
HMGR	3-hydroxyl-3- methylglutaryl- coA -coenzyme A reductase
HPLC	High performance liquid Chromatography
IPP	Isopentenyl pyrophosphate
IPTG	Isopropyl-β-D-thiogalactopyranoside
kb	Kilobase (s)
kDa	Kilodalton (s)
KS	Ent-kaurene synthase
MEP	Methylerythritol phosphate
MS	Mass spectrometry
MiRNA	MicroRNA
mRNA	Messenger RNA
MVA	Mevalonate
NBS	Nucleotide-binding site
OD	Optical density

ORF	Open reading frame
PCD	Programmed cell death
PCR	Polymerase chain reaction
PR	Pathogenesis-related
qRT-PCR	Quantitative real-time PCR
RNA	Ribonucleic acid
RNAi	RNA interference
RT-PCR	Reverse transcriptase-PCR
SDS	Sodium Dodecyl Sulfate
SOC	Super optimal broth medium with catabolite repression
SDS-PAGE	SDS polyacrylamide gel electrophoresis
SE	Standard error
smRNAs	Small RNAs
siRNAs	Small interfering RNAs
STARTS	Stable Root Transformation System
TBE	Tris-borate-EDTA
TE	Tris-EDTA
VIGS	Virus-induced gene silencing
TRV	Tobacco rattle virus
TMV	Tobacco mosaic virus

1. Introduction

1.1 Plant roots

Cereal roots have specialized functions in anchoring the plant in the soil against uprooting and lodging by wind, uptake of water, nutrients, salt stress and protecting the root from pathogen attack or interacting with beneficial microorganisms (Jackson et al., 1999; Gregory, 2006). Leakage of nutrients into the rhizosphere provides a micro environment for growth of many microorganisms living in association with the plant root. These microbes exhibit saprophytic, biotrophic or necrotrophic life styles. Some of these rhizosphere microbes colonize roots endophtically and provide beneficial effects on plant growth and health (Whipps, 2001; Kogel et al., 2006), while some fungi (e.g. of the genus *Fusarium*) are causal agents of severe plant diseases such as crown root rot (Mcmullen et al., 1997; Jansen et al., 2005). In addition, roots are consistently subjected to abiotic stress including low nitrogen, salinity, drought and heavy metal toxicity (Witcombe et al., 2008). Moreover, roots also have been identified as entry point for leaf pathogens (Sesma and Osbourn, 2004; Marcel et al., 2010) even more highlighting the significance of the root for plant health. Because of this, knowledge and technology on protective cultivation methods, resistant germplasms, or chemical control are eagerly required (Imani et al., 2011). However, a major limitation in the identification of genes mediating root stress resistance is the availability of an efficient functional assay system prior to regenerating whole transformed plants.

1.2 Transformation of cereal crops

1.2.1 Transient transformation

Biotechnological approaches bear the potential to improve stress adaptation of crop plants (Mittler and Blumwald, 2010) in addition to classical breeding strategies. Functional analyses of plant proteins rely on mutational approaches and silencing and/or overexpression of respective genes as well as modified gene versions. Effective test systems, developed for model plants such as *Arabidopsis* or tobacco (seedlings and adult plants or mutant libraries), are widely missing for cereal crop plants (Imani et al., 2011). Transient transformation in plant is a valuable approach for functional genomics and promoter testing (Caroline and Huw, 2009). The candidate genes could be expected to be over-expressed or silenced which does not rely on the chromosomal integration of herterologous DNA. Physical methods, *Agrobaterium* sp. or viral vectors are the mainly methods used for foreign DNA delivery to the plant cell. In transient transformation systems, virus vectors from e.g. tobacco rattle virus (TRV) or tobacco mosaic virus (TMV) have been used to over-express or silence plant genes. In virus-based systems, a sense or antisense fragment of a given plant gene of interest is inserted into the genome of the virus. The manipulated virus is subsequently used to infect plants. Replication of this fragment in the plant will silence the respective plant gene and eventually provide information about its function. In addition, the Green Fluorescent Protein (GFP) is widely used in plants like *Nicotiana* species, tomato, pea, *Arabidopsis*, and sugar beet to study protein localization and thus to obtain information about protein functions **(**MacFarlane and Popovich, 2000). Though transient gene expression

provides a convenient alternative to stable transformation in analyzing gene function by virtue of its time and labor efficiency, it has some problems like instability of the foreign gene in the viral genome and the appearance of disease symptoms caused by the virus in plants. Particle bombardment, a biolistic approach, is a commonly used method for genetic transformation of plants and other organisms. Millions of DNA-coated metal particles are shot at target cells or tissues using a biolistic device or gene gun (Kikkert er al., 2004). This method with a relatively simple protocol can be used to transform all plant species and no binary vectors are required. However it has some disadvantages e.g. (i) difficulty in obtaining single copy transgenic events, (ii) high cost of the equipment and microcarriers, (iii) intracellular targeting is random (cytoplasm, nucleus, vacuole, plastid, etc.), (iv) transfer DNA is not protected, and, most importantly, (v) a low transformation frequency in a given tissue (e.g. 15-20 transformed epidermal cells per expanded barley leaf). Based on recently developed *Agrobacterium tumefaciens*-mediated transfection (Agroinfiltration) combined with the virus vector, such as TMV or TRV, these disadvantages have been partly overcome (Marillonnet et al., 2005). However, transient transformation systems have not yet been established for cereal roots such as barley and wheat. Moreover, transfection-based methods are not suitable for cereal crop plants.

1.2.2 Stable transformation

Stable transformation technology shows significant role in modern plant biology which is not only used for the production of superior crop varieties, but also used for solving the basic questions concerning gene functions. The principal method for

stable plant transformation is *Agrobacterium tumefaciens*-mediated gene transfer (Estrella et al., 1983). *A. tumefaciens* could invade a large host spectrum (Newell, 2000) and naturally transfers the well-characterized transferred DNA (T-DNA), which is part of the bacterial tumor inducing (Ti)-Plasmid, into the plant genome (Tzfira, 2006). The gene of interest carried by the T-DNA is integrated into plant genomes which permit to study the function of recombinant proteins in various aspects of plant developmental and in responses to abiotic or biotic stress. The conventional transformation method used for Arabidopsis is *Agrobacterium*-mediated floral dip transformation (Clough and Bent, 1998), while leaf discs are used to transform tobacco and to regenerate transgenic plants (Horsch et al., 1985). A recently published review has summarized the various procedures currently available for transformation in the Triticeae (including barley,wheat, triticale, rye) (Kumlehn and Hensel, 2009). The transformation procedures used in tissue culture could be divided into three steps: First, select and prepare the explants that could be regenerated into adult plants; Second, choose the method for the foreign DNA transformation; Third, induce and regenerate callus and select the positive callus (Caroline and Huw, 2009). The commonly used transformation methods for the Triticeae are microinjection and *Agrobacterium*-mediated transformation. Different explants including isolated ovule, immature embryo, embryogenic pollen culture are used for transformation. Stably transformed wheat was first achieved by bombardment the embryogenic callus with the metal particles coated in plasmid DNA (Vasil et al., 1992). These days, barley is almost exclusively transformed by co-cultivation of the scutellum of immature

embryos (Tingay et al., 1997) as well as of androgenetic pollen (Kumlehn et al., 2006) with *A. tumefaciens* carrying the gene of interest. However, 12 months are required from the primary transformation event to the obtaining of T2 homozygous plants. Taken together, these systems are efficient but time-consuming for root-related functional studies. It underlines the requirement of fast and robust root transformation system for monocotyledonous plants to examine root development or root immunity and root-microbe interactions.

1.3 Root -microbe interactions

Plants roots are confronted with a plethora of microorganisms (pathogenic virus, bacteria or fungi) that aim to acquire nutrients. These interactions can be neutral, harmful (pathogenic), or beneficial (mutualistic symbiosis) to the host (Shen et al., 2006; Thrall et al., 2007). Interestingly, plants roots are resistant against most of the attackers and have evolved a series of defense mechanisms. In order to avoid pathogenic infection, plants have a two layered immune system. The first level of defense system depends on the recognition of conserved microbial structures, so called micro-associated molecular patterns (MAMPs), which is termed as MAMP-triggered immunity (MTI). The second level is defined as effectors-triggered immunity (ETI), which describes the recognition of virulence factors named effectors by resistance proteins (Jones and Dangl, 2006). Despite the fact that plant roots are surrounded by a biologically active zone rich in microorganisms, plant root–microbe interactions are poorly characterized (Singh et al., 2004). A number of evidences

showed that roots respond to various MAMPs, whereas the pathogenic micros have evolved mechanisms to suppress these responses (Millet et al., 2010).

The invasion strategies of pathogens differ in dependency of their life styles. For instance, biotrophic pathogens feed on living tissues and keep therefore the host alive, such as certain fungi (e.g. *Golovinomyces orontii*), oomycetes (e.g. *Peronospora parasitica*), and bacteria (e.g. *Pseudomonas syringae*) as well as all viruses. Necrotrophic pathogens such as the fungi *Alternaria brassicola* and *Botrytis cinerea* or the bacterium *Erwinia carotovora* kill host cell prior to or during invasion. They often release toxins or tissue-degrading enzymes to repress the plant defense system and to achieve the quick uptake of nutrients from plants (Staples, 2001; Rojo et al., 2003; Agrios, 2005). Hemibiotrophic pathogens, such as *Fusarium graminearum* which is a ubiquitous filamentous fungus, conceive an initial biotrophic infection phase but become necrotrophic during later interaction phases (Perfect and Green, 2001; Glazebrook, 2005; Ding et al., 2011). In mutualistic associations, the interaction state is thought to be well-balanced though host defense reactions are activated and disease could be observed (Kogel et al., 2006). However, in general, mutualistic interactions, do not disturb root function and development. Examples for such mutualistic microbes are mycorrhizal fungi and endophytes.

1.4 The mutualistic fungus *Piriformospora indica*

1.4.1 *P. indica*'s beneficial functions symbioses with host plants

Piriformospora indica was first discovered in 1990s in association with a spore of the AM fungus in the rhizospheres of woody shrubs in the sandy desert soils of the Thar region in India (Verma et al., 1998). *Piriformospora indica* was classified as member of the order Sebacinales, which belongs to the class of agaricomycetes. *Piriformospora indica* is a root colonizing basidiomycete that colonizes a broad spectrum of plants. The fungus conveys various beneficial effects to colonized host plants (Pham et al., 2004; Waller et al., 2005) that have been extensively studied in barley and Arabidopsis (Waller et al., 2005; Deshmukh et al., 2006; Schäfer et al., 2007). It was recently reported that *P. indica* improves the uptake of nutrients by host plants. It was suggested that *P. indica* stimulates nitrate uptake as it induces the expression of nitrate reductase (NR) genes. Co-cultivation of tobacco seedlings with *P. indica* causes approximately 50% increase in NADH-dependent NR activity in the roots (Sherameti et al., 2005). In maize, the growth promoting effects of *P.indica* were proven to depend on a phosphate transporter of *P. indica* (*PiPT*), which is thought to mediate phosphate transport to the host plant (Yadav et al., 2010). Moreover, *P. indica*-inoculated barley plants exhibited enhanced grain yield and tolerance to abiotic stress in barley (Waller et al., 2005). Among the benefits obtained by plants colonized by *P. indica* are local resistance against necrotrophic and hemibiotrophic root pathogens (e.g. *Fusarium culmorum, Fusarium. graminearum, P. herpotrichoides, Colleotrichium sativus*) and systemic resistance against powdery

mildew fungi (Waller et al., 2005, Deshmukh et al., 2007, Stein et al., 2008). Considering the various beneficial effects caused by *P. indica*, the fungus is regarded to have significant agronomical and high ecological relevance (Weiss et al., 2004; Schäfer and Kogel, 2009).

1.4.2 Compatibility in plant root- *P. indica* interaction

P. indica has a broad host spectrum including gymnosperms and monocotyledonous as well as dicotyledonous plants which suggests that the fungus has developed efficient colonization strategies (Jacobs et al., 2011). A transcriptome analysis of *P. indica*-colonised barley roots suggested that the fungus suppresses plant defense and alters gibberellic acid (GA) metabolism (Schäfer et al., 2009). It was noticed that barley plants impaired in GA synthesis and perception showed a significant reduction in mutualistic colonization, which was associated with an elevated expression of defense-related genes. Consistent with this, Jacobs et al. (2011) showed that root MTI is able to efficiently restrict penetration and root colonization by *P. indica*. However, the fungus has developed strategies to suppress root MTI. Despite the progress made in characterizing plant root-*P. indica* associations, knowledge about plant components involved in the establishment or restriction of the mutualistic association are still limited.

1.4.3 Cellular colonization pattern of *P. indica*

Studies in barley roots demonstrated that *P. indica* colonization predominantly happened in the maturation zone while the fungus was almost absent in younger root

parts (e.g. meristem region) (Deshmukh et al., 2006). After spore germination extracellular colonization of the root takes place until 1-2 days after inoculation (dai) and hyphae frequently fuse and form an initial extracellular network. Around 3 dai, individual epidermal and cortex root cells are penetrated through the cell wall without formation of a specialized penetration organs (e.g. appressoria). As the interaction proceeds, colonization expands and at 7 dai parts of the root surface are covered while inter- and intracellular hyphae are abundantly found in the root epidermis and cortex (Deshmukh et al., 2006). Fungal sporulation starts as early as 7 dai (Schäfer et al., 2009). At 7 dai, a majority of hyphae were found in dead rhizodermal and cortical cells, and this was associate with the suppression of barley *BAX INHIBITOR-1* (Hv*BI-1*), a negative cell death regulator. Consistently, *P. indica* colonization was dramatically reduced in barley plants overexpresssing *BAX INHIBITOR-1* (Hv*BI-1*) (Deshmukh et al., 2006). These molecular analyses demonstrated that cell death emergence is required for *P. indica* proliferation. Recent studies demonstrated that *P. indica* obviously induces a vacuolar cell death by impairing endoplasmic reticulum function (Qiang et al., 2012). In sum, *P. indica* apparently follows a biphasic colonization pattern starting with an initial biotrophic phase during early cell colonization followed by a cell death-dependent colonization phase (Qiang et al., 2012).

1.5 Pathogenic fungus *Fusarium graminearum*

The pathogenic fungus *Fusarium graminearum* is a haploid homothallic ascomycete, which causes head blight on wheat, barley, and other grass species as well as ear rot

on corn (Bai and Shaner, 2004). The ascospores produced from fruiting bodies (perithecia) are able to germinate within 6 hours upon landing on susceptible parts of the host plant (Beyer and Verreet, 2005). Fusarium head blight (FHB) or scab of barley and wheat is considered the worst plant diseases which cause heavy economic losses to farmers due to yield decreases. In addition, the production of mycotoxin renders the grain useless for flour and malt products (Jansen, 2005). *F. graminearum* infects cereal florets and releases various hydrolyzing enzymes to facilitate colonization by direct penetration of the epidermal cuticle and cell wall (Walter et al., 2010; Ding et al., 2011). Plants have developed strategies, for instance cell wall reinforcement, accumulation of antimicrobial compounds, and a higher expression of pathogenesis-related (PR) genes, to ward off the attack (Pritsch et al., 2000; Li et al., 2001; Kang, 2003; Golkari et al., 2007). Recent studies indicated that early signaling events, for instance, oxidative burst, jasmonic acid (JA) and ethylene (ET)-dependent defense signaling are associated with plant colonization by *F. graminearum* (Geddes et al., 2008; Li, 2008; Jia et al., 2009). However, their exact roles and the coordinated regulations in FHB resistance in monocot (wheat, barley), and especially, in monocot roots, are unknown. Therefore, further investigations and efforts are required to improve the crop quality concerning the adaptability of such pathogens.

1.6 Two independent non-homologous metabolic pathways for the production of isopentenyl pyrophosphate (IPP) and dimethylallyl diphosphate (DMAPP)

Terpenoids, which play important role in cell metabolism and other aspects (physiological, structural functions) are a large group of natural compounds that are produced from the condensation of isopentenyl pyrophosphate (IPP) and dimethylallyl diphosphate (DMAPP) in all organisms (Rohdich et al., 2005; Withers and Keasling, 2007). Plant terpenoids are found among primary and secondary metabolites. Primary metabolites are necessary for cellular function and maintenance, for instance, including gibberellins, carotenoids and sterols (Toone, 2009), which serve as cell growth modulation and plant elongation. Some secondary metabolites in plant are used as toxic or repellant chemicals to support plant defense against herbivores and microbes (Rosenthal and May, 1991). The concentration of secondary metabolites increase when plants are attacked by herbivores or pathogens. When plants are attacked by insects, some compounds are even released into the air which attracts parasites and predators to kill the herbivores (Rosenthal and May, 1991). Recently, more primary roles of these chemicals, e.g. in signaling, antioxidation, have been identified in plants (Bidlack, 2000). There are two independent non-homologous metabolic pathways for the symthesis of (IPP and DMAPP): the mevalonate (MVA) pathway and the methylerythritol phosphate (MEP) pathway (Roberts, 2007) (Figure 1-1).

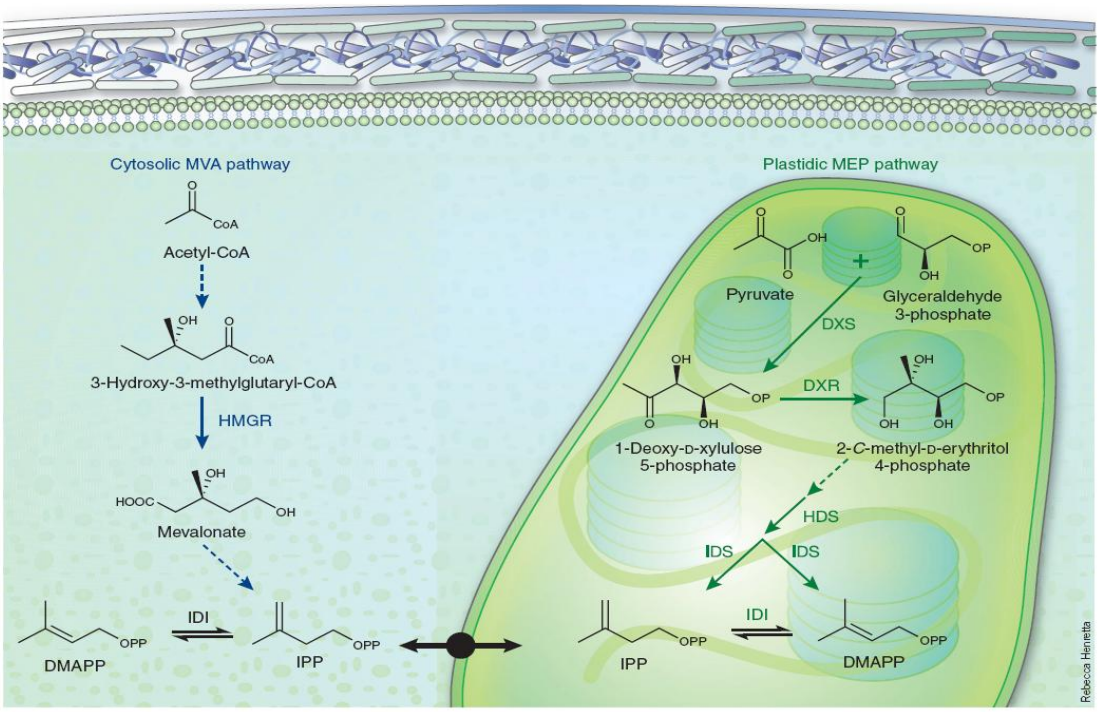


Figure 1-1. Compartmentalized biosynthesis of IPP and DMAPP. (Left) IPP and DMAPP produced via the cytosolic mevalonate (MVA) pathway. HMGR, 3-hydroxy-3-methylglutaryl, coenzyme A reductase; IDI, isopentenyl diphosphate isomerase. (Right) IPP and DMAPP synthesized via the plastidic MEP pathway. DXS, 1-deoxy-d-xylulose-5-phosphate synthase, DXR, 1-deoxy-d-xylulose-5-phosphate reductoisomerase; HDS, hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate synthase; IDS, isopentenyl diphosphate:, dimethylallyl diphosphate synthase; IDI, isopentenyl diphosphate isomerase. Dashed arrows indicate more than one step. (Taken from Roberts, 2007）

1.6.1 Mevalonate (MVA) Pathway

The mevalonate (MVA) pathway is an important cellular metabolic pathway present in all higher eukaryotes and many fungi. Interestingly, it seemed to be absent in most bacteria (Zhou and White, 1991). In the MVA pathway (cytosolic MVA pathway), three acetyl coenzyme A (CoA) are condensed into 3-hydroxyl-3- methylglutaryl-coA

which is catalyzed by the 3-hydroxyl-3-methylglutaryl-coA-coenzyme A reductase (HMGR) (Ha et al., 2003) to result in MVA (Roberts 2007). MVA is subject to a series of enzyme reactions to form the isoprenoid precursors IPP and DMAPP. By supplying the precursors for the production of sesquiterpenes and triterpenes, the MVA pathway plays key roles in the cytosol in plants, animals and fungi. However, there are still some differences of the MVA pathways between eukaryotes and archaea. (Figure 1-2) (Lombard and Moreira, 2011).

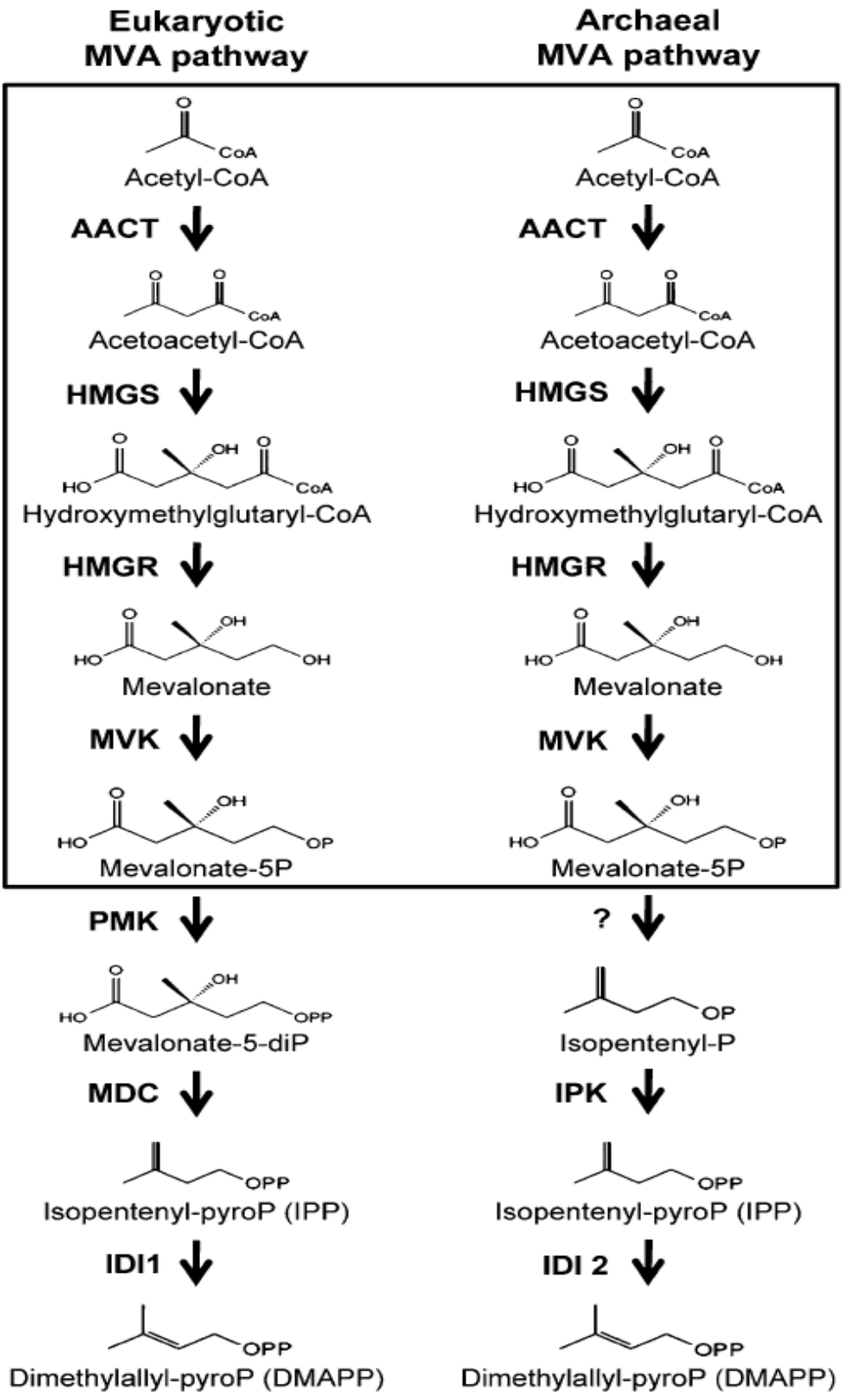


Figure 1-2. The partially different MVA pathways of eukaryotes and archaea.

The classical MVA pathway discovered in eukaryotes (left) and the new MVA

pathway proposed in archaea (right) only share the first four steps (boxed area). (Taken from Lombard and Moreira, 2011).

1.6.2 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway

Since the 1950s, the classic MVA pathway was considered as the sole source of the terpenoid precursors IPP and DMAPP (McGarvey and Croteau, 1995). The presence of another alternate pathway to synthesize IPP and DMAPP that originates from 2-*C*-methyl-D-erythritol-4-phosphate (MEP) was discovered in bacteria and plants in the 1990s (Rohmer, 1999; Eisenreich et al., 2001; Rodríguez-Concepción and Boronat, 2002; Phillips et al., 2008). It was recently confirmed that the MEP pathway is restrict to bacteria and plastid-bearing eukaryotes (Matsuzaki et al., 2008). In the MEP pathway, the substrate pyruvate and glyceraldehyde-3-phosphate are catalyzed by the enzymes DXP synthase (DXS) and DXP reductoisomerase (DXR) to form the 2-C-methyl-D-erythritol 4-phosphate, which serves as substrate for the synthesis of IPP and DMAPP by series of catalytic enzyme reactions (Roberts, 2007). Considering the central nature of the MEP pathway in these organisms, many researchers focused on the enzymes of the MEP pathway to develop novel antibiotics, antimalarials and herbicides (Ortmann et al., 2007; Eoh et al., 2009). Recent studies have demonstrated that there is an exchange of the intermediates between the cytosol and plastids (Bick 2003; Hemmerlin et al., 2003; Laule et al., 2003). However, the interaction between MVA and MEP pathways is still largely unknown.

1.7 Genes involved in the GA metabolism

Gibberellins (GA) comprise a large hormone family in plants, whose synthesis depends on IPP and DMAPP. GAs modify many aspects including plant growth and development (Hedden and Kamiya, 1997; Hedden and Phillips, 2000; Hedden, 2001). For example, GA application accelerates flowering in Arabidopsis, and the GA-deficient mutant *ga1* is unable to flower (Sun and Kamiya, 1994). Moreover, a previous study indicated that GA produced in the developing anthers are required for corolla development (Weiss et al., 1997). In several plants, GA deficiency leads to male sterility because of the abnormal anther development (Goto and Pharis, 1999). Functional characteristic of GA-deficient mutants also revealed that GAs are required for seed development at least in several species, for instance, in tomato and in pea (Groot et al., 1987; Swain et al., 1997). It is further suggested that GAs are required for normal pollen tube growth (Singh et al., 2002). GA biosynthesis can be divided into three steps that encompass the following enzymes (Figure 1-3): (1) terpene cyclases, ent-copalyl diphosphate synthase (CPS) and ent-kaurene synthase (KS), (2) cytochrome P450 monooxygenases, including ent-kaurene oxidase (KO) and ent-kaurenoic acid oxidase (KAO), and (3) 2-oxoglutarate-dependent dioxygenases, including 20-oxidase (20ox) and 3-oxidase (3ox) (Sakamoto et al., 2004; Spielmeyer et al., 2004). Briefly, the substrate geranylgeranyl diphosphate (GGDP), generated from IPP and DMAPP, is converted to ent-kaurene via the diterpene cyclases, CDP synthase (CPS) and ent-kaurene synthase (KS), in plastids. Thereafter, the ent-kaurene is modified by the cytochrome P450 monooxygenases, ent-kaurene oxidase (KO) and

ent-kaurenoic acid oxidase (KAO) to produce GA_{12} via ent-kaurenoic acid. The final stage of bioactive GA synthesis, from GA_{53}/GA_{12} to GA_1/GA_4, is catalyzed through GA20-oxidase (GA20ox) and GA3-oxidase (GA3ox) in the cytosol.

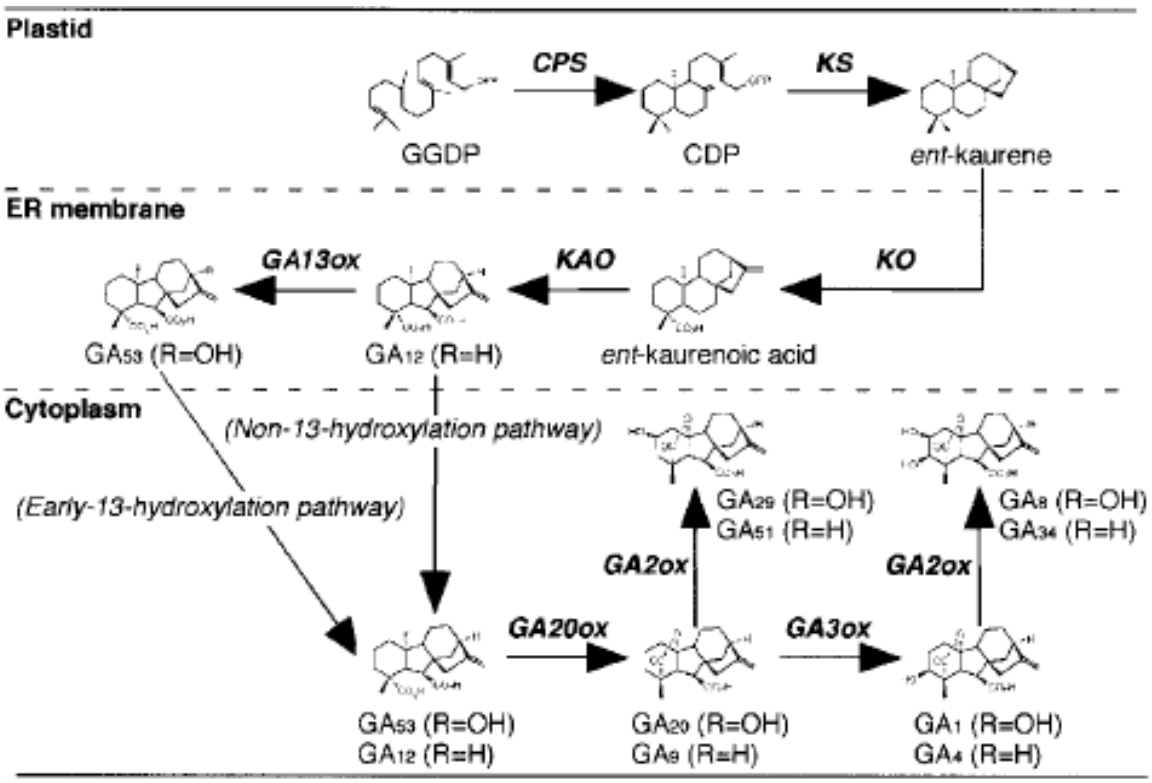


Figure1-3. Principal pathway of GA metabolism in plants. (Taken from Sakamoto et al., 2004).

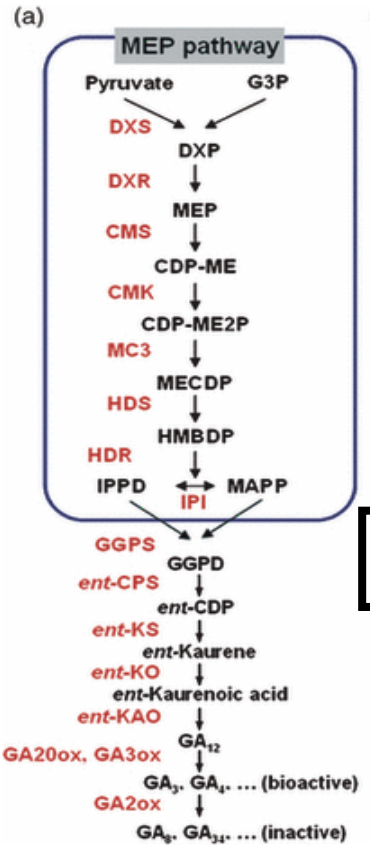
1.8 Regulation of members of the MEP and GA pathway in barley roots by *Piriformospora indica*

Barley belongs to the Poaceae family that includes wheat (*Triticum aestivum*), rice (*Oryza sativa*), and maize (*Zea mays*). Barley is a self-pollinator and has a diploid genome (2n=14) which is recognized as a genetic model within the Poaceae. In order to

speed up genomic research in barley, a multinational collaboration, so called international barley sequencing consortium (IBSC), aimed at obtaining the whole barley genome sequence (Schulte et al., 2009). Recently, more than 22,651 representative cDNA sequences were obtained (Matsumoto et al., 2011). Moreover, another estimated 32,000 barley genes are assigned to individual chromosome arms (Mayer et al., 2011). Genome, transcriptome and proteome studies of barley will deepen our understanding of the molecular function barley genes (Sreenivasulu et al., 2008a; Sreenivasulu et al., 2008b).

An earlier study showed that all genes involved in the MEP pathway are induced by *P. indica* (Figure 1-4) (Schäfer et al., 2009) as well as genes immediately lying downstream of the MEP pathway. For instance, the gene encoding a putative geranylgeranyl diphosphate synthase (GGPS), which catalyze the conversion of IPP and DMAPP into geranylgeranyl diphosphate (GGDP), was induced at 3 and 7 day after inoculation (dai). In addition, downstream of GGPS, two kaurene synthase genes (*ent-KS1a and ent-KS-like 4)* were differentially regulated in *P. indica*-colonized barley roots at 1, 3 and 7 dai. As mentioned above, the terpene cyclases, copalyl diphosphate synthase (CPS) and kaurene synthase (KS), play key roles in GA biosynthesis (Otomo et al., 2004). However, terpene synthases form a large gene family that are involved in the synthesis of a number of different compounds. Several members of the family of kaurene synthase-like genes (*OsKSL*), for instance, *OsKSL1*, *OsKSL3*, *OsKSL4*, *OsKSL5*, *OsKSL6*, *OsKSL7*, *OsKSL8* and *OsKSL11*, have been found in the rice (*Oryza sativa*) genome and suggested to be involved in diterpenoid

biosynthesis (Xu et al., 2007). In addition, some of these OsKSL enzymes are involved in phytoalexin biosynthesis and upregulated during rice defense responses. These finding provides a good foundation to elucidate the functions of terpene cyclases in barley. Moreover, I do not know, why does *P. indica* affect terpene cyclases in barley, and what is their function in barley.



(b)

Gene	1 dai	3 dai	7 dai
DXS1		11,3	14,0
DXS2		2,80	3,30
DXR		4,6	4,8
CMK1		2,5	2,4
CMK2		2,3	2,3
HDS1		5,4	4,50
HDS2		4,0	5,4
HDR		3,1	3,10
GGPS		2,5	3,20
ent-KS1a	6,1	12,4	10,7
ent-KS-like 4		14,2	17,3
GA2ox1		−2,3	

Figure 1-4. *Piriformospora indica* induces the methylerythritol phosphate (MEP) pathway in barley. (Schäfer et al., 2009).

1.9 Terpene cyclase in developmental processes

In rice, 15 compounds have been identified as phytoalexins including oryzalexin A-F, momilactones A and B, oryzalexin S, and phytocassanes A–E. Almost all of these phytoalexins as well as the phytohormone GA are diterpenes commonly biosynthesized from geranylgeranayl diphosphate (GGDP) (Otomo et al., 2004). Here, the ent or syn-copalyl diphophate synthase (CPS) catalyze the conversion of GGDP to ent-CDP or syn-CDP, respectively. These are two stereoisomers due to a different protonation at the C-terminus of GGDP. Subsequently, ent-CDP or syn-CDP is converted into kaurene via kaurene synthase-like enzymes by the elimination of the phosphate group from the GGDP (Figure 1-5). Dependent on the substrates a plethora of different enzymes are involved in the synthesis of the various terpenoids. In *Arabidopsis*, the genes *GA1* to *GA5* encode ent-kaurene synthase A (now named copalyl diphophate synthase), ent-kaurene synthase B, ent-kaureneoxidase, GA 3-oxidase and GA-20 oxidase, respectively (Sun et al., 1992; Chiang et al., 1995; Xu et al., 1995; Helliwell et al., 1998; Yamaguchi et al., 1998). Mutants lacking one of these five genes (*ga1*-*ga5*) are blocked at different enzymatic steps of the GA biosynthesis pathway, which result in dwarf or semi-dwarf phenotypes. In wheat, three cDNAs encoding the ent-copalyl diphosphate synthases *TaCPS1, TaCPS2, and TaCPS3* were cloned, and the phylogenetic and expression analyses suggested that *TaCPS3* is responsible for gibberellin biosynthesis, while *TaCPS1* and *TaCPS2* are possible functional homologs of diterpene cyclase genes *OsCPS2* and *OsCPS4* that are involved in phytoalexin biosynthesis in rice (Tomonobu et al., 2009). However, so far, there is no report about

functional analysis of terpene cyclase in barley. A previous work reported on the isolation of barley Hv*KSL1* and Hv*CPSL1* genes without giving further information on putative functions (Spielmeyer et al., 2004). Concerning *ent-KS1a* and *ent-KS-like4* which are induced by *P. indica* it is unknown whether these genes are involved in GA biosynthesis or other terpenoid metabolites.

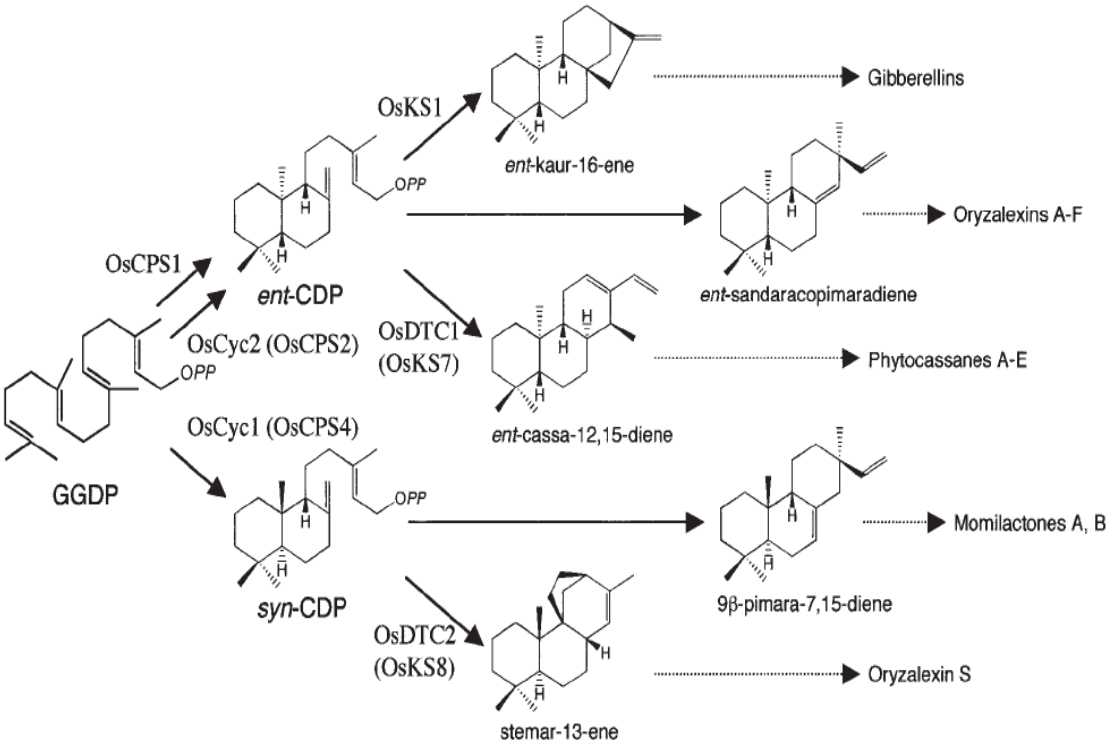


Figure 1-5. Proposed biosynthetic pathway of the diterpene phytoalexin in rice. (Taken from Otomo et al., 2004).

2.0 Objectives

To understand the function of proteins involved in the establishment of mutualistic and pathogenic plant root-microbe interactions, a fast and more efficient method is urgently required. The aim of the present study is to develop a transformation method for barley roots that is as robust as conventional transformation methods but is more time-efficient in the generation of transformed roots. Moreover, the system should enable me to

1) perform gene silencing and overexpression,

2) conduct protein localization studies at sub-cellular resolution,

3) analyze protein functions in pathogenic and symbiotic barley root-microbe interaction.

The second central interest of my project is to determine the effect of *ent-KS1a* and *ent-KS-like4*, which are induced by *P. indica* (Schäfer et al., 2009), on root colonization by *P. indica*. The barley genome contains a large kaurene synthase gene family which forms the key branch genes involved in the biosynthesis GA and other diterpenoids. It is unknown, whether both genes induced by *P. indica* are involved in GA synthesis or the production of other diterpenoids. A knock down of these genes might reveal dwarf phenotypes as reported for GA synthesis mutants thereby suggesting a role of these genes in GA metabolism. Therefore, the second aim of my work is to use the stable root transformation method to analyse

1) *P. indica* colonization of roots in which either gene is knocked down,

2) regenerate stable transgenic plants silenced in either gene to study any growth phenotypes,

3) the impact of *P. indica* on *KS-like* gene expression and biosynthesis of terpenoids.

2. Materials and Methods

2.1 Plant, plasmids and fungal material

2.1.1 Barley (*Hordeum vulgare* L.)

Barley (*Hordeum vulgare* L.) cv. Golden Promise (GP) and GP stably overexpressing the Green Fluorescent Protein (Schultheiss et al., 2005) were used in this study for immature embryo isolation. Plants were grown in a climate chamber at 18℃/14℃ (light/dark) with 65% relative humidity, a 16 h photoperiod and a photon flux density of 240 μmol m^{-2} s^{-1}.

2.1.2 Vectors and gene plasmids

For the study, mGFP5-ER (Haseloff and Siemering, 2005) and pIPK007 (Himmelbach et al., 2007) vector were provided by Jim Haseloff (University of Cambridge, UK) and Jochen Kumlehn (IPK Gatersleben, Germany), respectively. The silencing vector p7U-35S-RNAi and p7i-Ubi-RNAi vector were provided by DNA-Cloning service (www.dna-cloning.com). The barely kaurene synthase-like (Hv*KSL*) gene plasmids Hv*AK370792*, Hv*syn-CPS* and Hv*KSL1*, Hv*ent-CPSL1* were provided by Takashi Matsumoto (National Institute of Agrobiological Science, Japan) (Matsumoto et al., 2011) and Peter M. Chandler(CSIRO. Plant industry, Australia) (Spielmeyer et al., 2004), respectively.

2.1.3 Fungal strains

In this study, *Fusarium graminearum* wild type (strain 1003) (Babaeizad et al., 2008), and *Piriformospora indica* DSM11827 (German collection of microorganisms and cell

cultures in Braunschweig, Germany) were used throughout the investigation. *F. graminearum* wild type (strain 1003) was routinely cultured on SNA (synthetic nutrient poor agar) plates (see below). The plates were incubated at room temperature and under constant illumination from one near-UV tube (Phillips TLD 36 W/08, www.philips.de) and one white light tube (Phillips TLD 36 W/830HF, www.philips.de). The isolate of *P. indica* DSM11827 (German collection of microorganisms and cell cultures in Braunschweig, Germany) was isolated at the Indian Thar desert in 1990s (Savita et al., 1998). The chlamydospores of *P. indica* were stored in glycerin at -80 ℃, which was used to prepare master plates, which served as basis to propagate the fungus on plates containing modified *Aspergillus* minimal medium (see below, modified after (Pham et al., 2004) at 22 ℃ for 6 to 8 weeks.

SNA (synthetic nutrient poor agar)

1 g KH_2PO_4
1 g KNO_3
0.5 g MgSO4 x $7H_2O$
0.5 g KCL
0.2 g glucose
0.2 g sucrose
15 g Agar. Add 1 liter H_2O_{dest}

CM medium (Modified *Aspergillus* minimal medium)

50 ml 20 x Salt solution
20 g Glucose
2 g Peptone
1 g Yeast extract

1 g Casamino-acid

1 ml Microelements

Add 950 ml H_2O_{dest}

Optional: 15 g Agar

20 x Salt solution

120 g $NaNO_3$

10.4 g KCl

10.4 g $MgSO_4$ x $7H_2O$

30.4 g KH_2PO_4

Add 1 liter H_2O_{dest}

Microelements

6 g $MnCl_2$ x $4H_2O$

1.5 g H_3BO_3

2.65 g $ZnSO_4$ x $7H_2O$

750 mg KI

2.4 mg Na_2MO_4 x $2H_2O$

130 mg $CuSO_4$ x $5H_2O$

Add 1 liter H_2O_{dest}

2.2 Molecular cloning and plasmids constructions for STARTS

For overexpression of *GFP*, *GFP-BI-1*, and for localization of *mGFP5-ER* in barley roots by STARTS, the binary vectors pLH6000-Ubi::*sGFP* (synthetic GFP, GenBank accession no. AM261415), pLH6000-Ubi::*sGFP-BI-1* (Deshmukh et al., 2006), and pLH6000-35S::*mGFP5-ER* were used, respectively. mGFP5-ER was previously published (Haseloff and Siemering 1998). For silencing of Hv*EXPANSIN B1*， a 155 bp sequence was amplified using the primers (5'-CAAGCAACGAACACCGAGT-3', 5'-GGCTTACGAAGAGCGATAGG-3') and cloned into the entry vector pENTR-D-TOPO (Invitrogen, www.invitrogen.com). Primers used in this part were ordered from Eurofins MWG Operon. The Hv*EXPANSIN B1* segment was then inserted into the destination vector pIPKb007 (Himmelbach et al., 2007) substituting for the Ccdb gene using the LR reaction (Gateway system, Invitrogen,

www.invitrogen.com). For *GFP* silencing a 280 bp of the coding sequence of *GFP* (from 430 bp to 710 bp) was cut off by *Bsp*1407I and *Bam*HI from pGY1-GFP vector and cloned into the p7U-35S-RNAi vector (DNA-Cloning service, www.dna-cloning.com). Primer design was performed with the online tool Primer 3 (http://frodo.wi.mit.edu/primer3/). Restriction sites were introduced on 5' ends of the primers to facilitate cloning when necessary. The freeware pDRAW32 (http://www.acaclone.com/) was used for vector information management and *in silico* cloning. During cloning, the digestion was performed with restriction enzymes from Fermentas (Fermentas, Sankt Leon-Rot). Selection of suitable reaction buffer systems for double digestion was assisted by the online tool Double Digest™ (http://www.fermentas.com/en/tools/doubledigest). For the respective plasmid transformation and identification in the *E. coli* cell, standard transformation procedures were used as followed: competent cells of the strain *DH5α* (200 µL per tube) was thawed on ice, max. 20 µL of a ligation reaction was added and mixed very gently. The tubes were incubated on ice for 30 min. Subsequently, heat shock was performed for 60 sec to 2 min at 42 ℃ and the tubes were placed immediately on ice for at least 2 min. 800 µL SOC medium (see below) were added to each tube and incubated for 1 hour at 37 ℃ with vigorously shaking. Afterwards, most of the supernatant was removed and the cell pellet was resuspended with the rest SOC medium in the tube by pipetting. The suspension was plated on a LB agar plate containing the appropriate antibiotic. The plates were incubated overnight at 37 ℃. Positive colonies were selected on antibiotic selection plates.

SOC medium

2%	Tryptone
0.5%	Yeast Extract
10 mM	NaCl
10 mM	$MgSO_4$
10 mM	$MgCl_2$

2.3 Vector construction to overexpress and silence Hv*KS-like* genes

2.3.1 Standard Polymerase Chain Reaction (PCR) for the amplification of genes of interest

Full length genes and gene fragments of Hv*KS -like* genes were amplified by standard PCR in a 20 µl volume. The reaction mixture contained 0.2 µl Phusion^{TM} high-fidelity DNA polymerase (New England Biolabs, UK), 4 µl 5 X high-fidelity (HF) buffer, 10 mM dNTPs (Amersham Pharmacia Biotech, Freiburg, Germany) 0.4 µl, 10 pmol of respective forward and reverse primers together with 20 ng DNA template. The standard PCR amplification was preceded in TProfessional cyclers (Biometra, Göttingen, Germany). The PCR scheme is described below. Afterwards, the PCR products were mixed with 10 X DNA-loading buffer and then separated by gel electrophoresis. According to the size of PCR products, the concentration of TAE-gels was ranged from 1-2% containing 1% (v/v) ethidiumbromid (stock solution: 10 mg/ml). Subsequently, the visualisation of PCR products on the gel was performed with a UV-Transluminator (Fröber Larbortechnik, Lindau, Germany) at a wavelength of 312 nm. All results were documented by video documentation equipment (digitStore, INTAS, Göttingen, Germany).

PCR Scheme

Denaturation	95℃	5min	
Denaturation	95℃	30 s	
Annealing	55-60℃	30 s	30-35 cycles
Elongation	72℃	45-120 s	
Final elongation	72℃	10 min	
Reaction termination	4℃	Pause	

10 X TAE

$C_4N_{11}NO_3$	242 g Tris
Acetic acid	57.1 ml
0.5 M EDTA	100 ml
pH: 8.5	

10 X DNA-Loading buffer

Bromphenol blue

40% Sucrose

2.3.2 p7i-Ubi–HvKSL1/ HvAK370792-RNAi silencing vectors construction

For Hv*KSL1* and Hv*AK370792* silencing, a 242 bp and 300 bp fragment of the coding sequence were cloned into the p7i-Ubi-RNAi vector (DNA-Cloning service, www.dna-cloning.com). The restriction sites *Hind*III, *Bam*HI (underlined bp in sequences, see below) were added to the gene specific primers. Amplification was achieved using the Phusion™ high-fidelity DNA polymerase (New England Biolabs, UK). The fragments were first cloned into pGEM-T easy vector (Promega, Madison, USA*)* and sequenced by LGC Genomics (Berlin, Germany). The fragments were then released by

respective restriction enzyme (depending on used primers) digestion and ligated into the destination silencing vector p7i-Ubi-RNAi. The empty vector of p7i-Ubi-RNAi was constructed by removing the GUS fragment. p7i-Ubi-Hv*KSL1*-RNAi and p7i-Ubi-Hv*AK370792*-RNAi were transformed into the *Agrobacterium* strain AGL-1 by electroporation using an *E. coli* Pulser™ transformation apparatus (Bio-Rad Laboratories, Hercules, CA, USA) following the manufacturer's instruction. Briefly, the 0.2 cm *E. coli* Pulser electroporation cuvette was chilled on ice. 1 μL aliquots of plasmid DNA (about 100 ng) was mixed with 50 μL of electro-competent AGL-1 cells by pipetting up and down. After 1 min incubation on ice, the *E. coli* Pulser™ apparatus (Bio-Rad) was set to a voltage of 2.5 KV for transformation. 1 mL SOC medium was added to the cuvette and incubated at 28℃ for 2 h. The cells were plated on YEB medium (see below) containing 25 μg/mL Rifampin, 25 μg/mL Carbenicillin and 50 μg/mL Spectinomycin. 2 days later, positive colonies were confirmed by PCR and selected for liquid culture. Miniprep extraction of the plasmid was performed using NucleoSpin® Plasmid DNA purification kit (MACHEREY-NAGEL GmbH, Düren, Germany). Plasmids were confirmed again using respective restriction enzyme digestion.

HvAK370792 /Silent-F: AAGCTTGAGGCCAGGATAAGGAAGCAG
HvAK370792 /Silent-R: GGATCCGAAGAGAAAGTGAGATTGTAGCCA

KSL1/Silent-F: AAGCTT GTTGCACCGGAATCGGATTC
KSL1/Silent-R: GGATCCGCAGTATCCATTCAACACATTGG

KS2/Silent-F: AAGCTT GCTTGATTAACCTGCCCACT
KS2/Silent-R: GGATCC TTGTCGGAAGATGGTGCCTG

KS4/Silent-F: AAGCTT TCGTCACCTTCTCCGAGACA
KS4/Silent-R: GGATCC CATGGATACCCTTGTGGATGTT

YEB medium

5 g Beef Extract

1 g Yeast Extract

5 g Peptone

5 g Sucrose

0.5 g $MgCl_2$ To prepare YEB plates, add bacterial agar to 15 g in 1 liter.

2.3.3 pET-30a (+)-Hv*KS-Like* gene overexpression vector construction

To analyze barley KS-like enzyme function, the open reading frame (ORF) lacking the transit peptide region was amplified by PCR using the Phusion™ high-fidelity DNA polymerase (New England Biolabs, UK). Primers used for cloning full length cDNA of Hv*KSL1*, Hv*AK370792*, Hv*ent-CPS* and Hv*syn-CPS* as well as the accession number for these genes are listed in Table 2.1. The restriction sites *Eco*RV and *Xho*I for Hv*AK370792*, *Bgl*II and *Xho*I for Hv*KSL1*, *Bgl*II and *Eco*RI for Hv*ent-CPS* and Hv*syn-CPS* were added to the primer and used for expression vector cloning. PCR amplified fragments were subcloned into the pGEM-T vector system (Promega, Madison, USA) according to the manufacturer's instructions and sequenced using M13 forward (GTTGTAAAACGACGGCCAGT) and M13 reverse primers (CAGGAAACAGCTATGACC) by LGC Genomics (Berlin, Germany). After having verified the sequence identity, the coding sequences were subcloned into pET30a (+) using the above listed restriction sites. The vector pET30a (+) allows expression of the protein of interest with an

N-terminal His_6-tag. Expression strain *E. coli* BL21 (DE3) was used bearin

g the additional plasmid pLysS for suppression of leaky expression of the target gene. Empty vector pET30a (+) was used as control.

Table2.1. Gene accession numbers and PCR primer sequences

Gene	Accession number	Primer	Sequencing
Hv*AK370792*	AK370792	HvAK370792 *Eco*RV-F	5'-GATATCATGTATGTCGAGAGCAGGCCGT-3'
		HvAK370792-*Xho*I-R	5'-CTCGAGATTTTCCAATTGAGCAGACA-3'
Hv*KSL1*	AY551436	HvKSL1-*BglII*-F	5'-AGATCTATGTGCGCCAGCGTCG-3'
		HvKSL1-*XhoI*-R	5'-CTCGAGCTAACTTGAGAGCCTGAGTGG-3'
Hv*ent-CPS*	AY551435	Hvent-CPSL-*BglII*-F	5'-AGATCTATGCTCGCCGTCAAAGGT-3'
		Hvent-CPSL1-*Eco*RI-R	5'-GAATCCTCAAAACACATGTTCGAAAATG-3'
Hv*syn-CPS*	AK364238	Hvsyn-CPS-*BglII*- F	5'-AGATCT ATGCTGACATTTACCGCTGC-3'
		Hvsyn-CPS-*Eco*RI -R	5'-GAATTC CTAAATCACATCCTGAAATATGAC-3'

2.4 Stable root transformation system (STARTS) for the generation of barley roots

For barley transformation, the above mentioned vectors were introduced into *Agrobacterium tumefaciens* strain AGL-1 by electroporation (*E. coli* Pulser, Bio-Rad, www.bio-rad.com*).* Two weeks post anthesis, spikes from barley plants were harvested. After removing awns, kernels were put in a bottle and placed on ice. For each transformation 100-200 kernels were surface sterilized in 70% ethanol for 5 min and subsequently incubated in sodium hypochlorite (3% active chlorine) for further 20 min. The kernels were washed once with sterilized water (pH 3) and then rinsed 3 times with sterile distilled water under sterile conditions. Immature embryos were taken from the caryopses, and the embryonic axis was removed with a sharp scalpel using a binocular microscope (Figure 2-1). Immediately, the obtained scutella were placed upside down onto callus induction medium (BCID, Table 2.2) (Tingay et al., 1997). 25-30 scutella were collected in the middle of a petri dish on callus induction medium. 200 µl overnight *Agrobacterium tumefaciens* culture (OD = 0.6) was added drop wise onto the scutella. Thereafter, scutella were turned again (now downside-up) and co-cultivated at 24 ℃ for 40-60 minutes in the dark. Thereafter, 10-12 scutella were transferred to a new plate and co-cultivated under the same conditions for 48 h. To support the preferential formation of transgenic callus and to remove the persisting *Agrobacteria*, the scutella were cultured on BCID medium supplemented with 50 mg L^{-1} hygromycin and 150 mg L^{-1} ticarcillin / clavulanate (1:15). The calli were subcultured at an interval of 2 weeks under the same conditions until roots were harvested. For root induction transgenic calli

were transferred into modified root induction medium (Jensen 1983) (Table 2.3). Root development was observed within two weeks. Transgenic barley overexpressing *synthetic GFP* (*sGFP*) under control of the CaMV35S promoter was generated by the conventional *A. tumefacines*-based transformation method as described by Tingay et al. (1997).

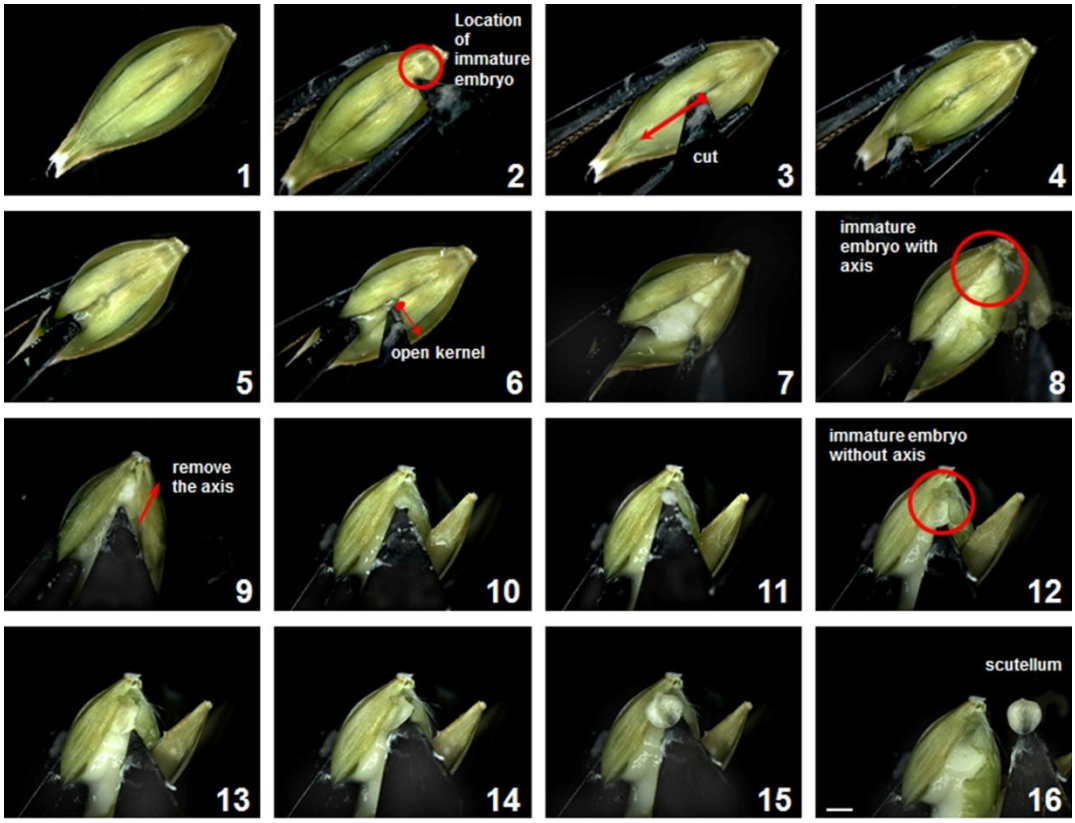


Figure 2-1. Steps for immature embryos isolation from barley.

Table 2.2.Composition of barley callus induction medium (BCID, 1 L)

MS-stock (Duchefa M0221)	4.3 g
$CuSO_4$ x $5H_2O$	1.2 mg
Maltose	30 g
Thiamin HCl	1 mg
Myo-inositol	250 mg
Caseinhydrosylate	1 g
L-Proline	690 mg
Dicamba	2.5 mg

Media should be filter sterilized

Add 5g l^{-1} autoclaved phytoagar (Duchefa)

After two subcultures at an interval of 2 weeks, calli were transferred to root induction medium (Table 2. 3) leading to emergence and growth of multiple roots from single callus cells within 2-3 weeks.

Table2. 3. Composition of barley root induction medium (1 L)

$CaCl_2$ x $2H_2o$	295 mg
KH_2PO_4	170 mg
KNO_3	2200 mg
MgSo4 x $7H_2O$	310 mg
NaH_2PO_4 x H_2O	75 mg
$(NH_4)_2SO_4$	67 mg
NH_4NO_3	600 mg
$COCL_2$ x $6H_2O$	0.025 mg
$CuSO_4$ x $5H_2O$	0.025 mg
H_3BO_3	3 mg
$MnSO_4$ x H_2O	5 mg
Na_2MoO_4 x $2H_2O$	0.25 mg
$ZnSO_4$ x $7H_2O$	5 mg
Fe-citrat x $5H_2O$	20 mg
Fe-EDTA***	28 mg
Nicotinamid	1 mg
Pyridoxine-HCL	1 mg
Thinmin-HCL	10 mg
Arginine	25 mg
Asparagine	50 mg
Asparaginic acid	30 mg
Glutamine	120 mg
Proline	50 mg
Threonine	25 mg
Pepton from Casein	125 mg
Myo-inosit	100 mg
Coconut milk*	25 ml
Glucose	7 g
Saccharose	20 g
Charcoal**	1 g

*Filter sterilization

** add after measurement of pH (5, 3)

Media should be filter sterilized

Add 5g l^{-1} autoclaved phytoagar (Duchefa)

2.5 Preparation of fungal inoculum and root inoculation

2.5.1 *Fusarium graminearum* inoculation

Conidial suspension was scratched from one-week-old plates by using sterile water and filtered through mira-cloth (Calbiochem, www.merck-chemicals.de) prior to the adjustment of conidia concentrations to 1.2×10^4 spores mL^{-1}. STARTS-generated roots (2.4) were inoculated with 1.2×10^4 spores mL^{-1} in 0.02% Tween 20 (v/v) + 0.5% gelatine (w/v) for 2 h. Thereafter, inoculated roots were transferred to agar plates. Root samples were harvested at 2 and 5 days after inoculation (dai) and subjected to DNA isolation, which was used for determining the amount of fungal DNA by quantitative Real Time-PCR.

2.5.2 *Piriformospora indica* inoculation

Piriformospora indica culture was maintained at 22℃ on complex medium (CM). Chlamydospores were collected from 6-week-old *P. indica* plates with sterilized Tween20-H_2O (1:5000). The spore concentration was adjusted to 5×10^5 chlamydospores mL^{-1}. For inoculation, STARTS-generated roots were immersed in an aqueous solution of 0.02% Tween20 containing 5×10^5 spores mL^{-1} for 2 h. Then the roots were transferred to ½ MS medium. For gene expression analyses barley seeds were surface sterilized in 6% sodium hypochloride for 2 h, dehusked and washed 3 times with sterile water under a sterile laminar flow. Afterwards, seeds were transferred to a jar containing PNM medium for germination. Three-day-old seedlings were used for inoculation with *P. indica* spore suspensions (5×10^5 spores mL^{-1} in 0.02%

Tween20). Root samples were harvested at 3 and 7 dai and subjected to DNA isolation, which was used for determining the amount of fungal DNA by quantitative Real Time-PCR (qRT-PCR).

½MS medium

4-5% gelrite

½ concentration of MS salts

PNM medium

1 M KNO_3	0.5 ml
KH_2PO_4	0.05 g
K_2HPO_4	0.025 g
1 M $MgSO_4$ x $7H_2O$	2.0 ml
1 M Ca $(NO_3)_2$	0.2 ml
0.02 M Fe-EDTA	2.5 ml
NaCl	0.025 g
Gelrite	4 g

pH 5.6 Add H_2O to 1liter, Autoclave.

2.6 Molecular biological standard methods for gene functional analysis

2.6.1 Extraction of genomic DNA and quantitative Real Time PCR

For colonization experiments,*P. indica INTERNAL TRANSCRIBED SPACER* (Pi*ITS*), *F. graminearum TUBULIN* (Fg*TUB*) and barley *UBIQUITIN* (Hv*UBI*) were amplified by qRT-PCR. To quantify fungal colonization levels, amplification of Pi*ITS* and Fg*TUB* were related to Hv*UBI* using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001).

Genomic DNA was isolated from 100 mg root tissue by using the DNeasy plant Mini Kit (Qiagen, www.qiagen.com) according to the manufacturer's instructions. For qRT-PCR, 10 ng of total DNA were used. Amplifications were performed in 7.5 µl of SYBR green JumpStart Taq ReadyMix (Sigma-Aldrich, www.sigmaaldrich.com) with 0.7 pmol oligonucleotides, using an 7500 Fast cycler (Applied Biosystems, www.appliedbiosystems.com). After an initial activation step at 95 ℃ for 5 min, 40 cycles (95 ℃ for 30 s, 60 ℃ for 30 s, 72 ℃ for 30 s, and 65 ℃ for 15 s) were performed. Respective melting curves were determined at the end of each cycle to ensure amplification of only one PCR product. Ct values were determined with the 7500 Fast software supplied with the instrument. The primers used for all analyses are listed below:

Hv*UBIQUITIN* (GenBank acc.no. M60175):

Forward 5'-ACCCTCGCCGACTACAACAT-3';

Reverse 5'- CAGTAGTGGCGGTCGAAGTG-3'

Pi*ITS* (GenBank accession no. AF 019636):

Forward 5'-CAA CAC ATG TGC ACG TCG AT-3'

Reverse5'- CCA ATG TGC ATT CAG AAC GA-3'

Fg*TUBULIN* (GenBank accession no. AY635186):

Forward 5'-GGTCTCGACAGCAATGGTGTT-3'

Reverse 5'-GCTTGTGTTTTTCGTGGCAGT-3'

2.6.2 RNA Extraction

For *GFP*, Hv*EXPANSINB1* and Hv*KSL* expression, RNA was extracted from barley roots. Barley roots inoculated with *P. indica* were harvested at 3 and 7 dai and immediately frozen in liquid nitrogen. Roots samples were crushed to fine powder in liquid nitrogen with mortars and pestles. 2 ml microcentrifuge tubes were filled with the powder to one third of the volume. 1 mL TRIZOL extraction solution was added to each sample. 0.2 ml of chloroform was added per 1 ml of TRIZOL reagent. Samples were vortexed vigorously for 15 seconds and incubated at room temperature for 2 to 3 minutes. Samples were centrifuged at no more than 12,000 g for 20 minutes at 4 ℃. Following centrifugation, the mixture separated into a lower red, phenol-chloroform phase, an interphase, and a colorless upper aqueous phase. RNA remained exclusively in the aqueous phase. The upper aqueous phase was transferred carefully without disturbing the interphase into a fresh tube containing 500 µL isopropanal and vortexed briefly. The volume of the aqueous phase was measured (the volume of the aqueous phase was about 60% of the volume of TRIZOL reagent used for homogenization). The mixture was incubated for 30 minutes at -20 ℃ to increase the RNA yield. Samples were centrifuged at 4 ℃ and 13500 rpm for 30 min. The supernatant was discarded and the pellet was washed with 70% ethanol prepared with H_2O_{DEPC}. The washing step was repeated and then the pellet was air dried under the clean bench. H_2O_{DEPC} was added to dissolve the pellet. RNA concentration was determined by a NanoDrop ND-1000 Spectrophotometer (peqLab Biotechnologie GmbH, Erlangen, Germany). The RNA integrity was verified on denaturing 1.5% agarose gel containing 5% formaldehyde in

MOPS buffer (20 mM MOPS, 5 mM sodiumacetate, 1 mM EDTA, pH 7.0). The gel was visualized with a UV transilluminator.

2.6.3 cDNA synthesis

RT-PCR was preformed with Qiagen Quantitect Reverse Transcription Kit. 1 µg RNA from each sample was added with a mixture containing 2 µL gDNA wipeout buffer withvariable RNase-free water to the final volume of 14 µL. After 2 min incubation at 42 ℃, 1 µg RNA was used for cDNA synthesis with 1 µL RT-primer mix, and 4 µL Quantiscript RT buffer and 1 µL Quantiscript reverse transscriptase . The reactions were incubated in a TProfessional thermocycler (Biometra GmbH, Germany) following the program of 42 ℃ for 60 min and 95°C for 3 min. Eventually, 80 µL MilliQ H_2O was added to each sample. Two aliquots were made from the cDNA samples and stored at -20 ℃ prior to use.

2.6.4 Real time PCR for checking the gene expression

In all cases, the barley housekeeping gene Hv*UBIQUITIN* was used as an internal control for equal loading. Transcription levels of *GFP*, Hv*EXPANSIN B1*, Hv*KSL1*, Hv*AK370792,* Hv*KS4* were determined via the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001) by relating the amount of target transcript to Hv*UBIQUITIN*. For qRT-PCR, the details were described in chapter 2.6.1. The primers used for all analysis are listed below:

Hv*EXPANSIN B1* (Genbank accession no. AY351786):

Forward 5'- CTGGTTCTGCAATTTGTGAG-3'

Reverse 5’-CTTTGCTGTGACTACAACTG-3’

Synthetic *GFP* (GenBank accession no. AM261415):

Forward 5’-ACCATCTTCTTCAAGGACGA-3’

Reverse 5’- GGCTGTTGTAGTTGTACTCC-3’

Hv*KSL1* (GenBank accession no.AY551436)

Forward 5’-GACAGCCAAGGCTTTGAGAG-3’

Reverse5’-TGCACATCTTCCAGAACAGC-3’

Hv*AK370792* (GenBank accession no.AK370792)

Forward 5’-TTCTCTTCTTTGCTCATCCTG -3’

Reverse5’-TCTTTCTATTTCCATCTCCCGA -3’

Hv*KS4* (GenBank accession no. AK251219)

Forward 5’-CGTCACCTTCTCCGAGACAT -3’

Reverse5’- GACGAACCTTCCTTGGGAGT-3’

2.7 GFP protein silencing efficiency detection

2.7.1 Root collection from STARTS and protein extraction

The GFP overexpressing roots transformed with the GFP-RNAi construct were generated 2-3 weeks after the callus was transferred to root induction medium. Different degrees of GFP silencing in roots were observed under the microscopy, separately collected according to the classifications not silenced, partially silenced and completely silenced. All harvested root samples were grinded to fine powder using liquid nitrogen. For each sample, around 100 mg root material was added to 400 µl

extraction buffer followed by two subsequent centrifugation steps at 1000 rpm for 15 min and at 15 000 rpm for 30 min. The supernatant was used for the analysis. Protein concentrations were determined using the Bio-Rad Protein Assay (Bio-Rad). Subsequently, the protein aliquots were stored at -20 ℃.

Protein extraction buffer

50 mM TRIS-acetate pH 7.4

10 mM potassium-acetate

1 mM EDTA

5 mM DTT

0.5 mM PMSF

2.7.2 SDS-PAGE and Western blot analysis of GFP protein silencing efficiency

20 µg of each protein sample was obtained using 5 X sample loading buffer (Pierce, Rockford, USA). Samples were denatured at 95 ℃ for 5 minutes and used for electrophoresis using acrylamide gels (1 mm, 10%). Stacking gel and separation gel were prepared as described below. The PAGE system was assembled and filled with 1 X SDS running buffer. The denatured samples were loaded on the gel and run at 100 V for ~1.5 h until bromophenol blue front reached the end of the gel. After electrophoresis, the gels were transferred to 1 X Towbin buffer.

For Western blot analysis, Roti-PVDF membrane (Roti®-PVDF, pore size 0.45 µm, ROTH, Germany) was cut and activated by methanol. Subsequently, the Western sandwich was assembled and put in cold 1 X Towbin buffer. After blotting (80 V for 2 h or 25 V overnight, 4 ℃), proteins were transferred to the PVDF membrane. The

membrane with transferred proteins was washed three times with PBST buffer for 10 min each time. Non-specific binding sites were blocked using 5 % (w/v) milkpowder (ROTH, Germany) in PBST buffer at 4 ℃ overnight. The PVDF membrane was immunostained with 1:4000 diluted mouse anti-GFP primary antibody (Molecular Probes, www.invitrogen.com) for 2 h at RT. The secondary antibody anti-mouse-HRP was applied at 1:10000 dilution for 1 h at RT. After washing the incubated membranes with 1 X PBST buffer (5 times, 5 minutes each), the detection was performed by using the ECL Plus Western Blotting Detection Kit for HRP (Amersham Biosciences, www.gelifesciences.com) on a Molecular Dynamics Storm 840 (Cambridge Scientific, www.cambridgescientific.com). Total protein load was indicated by Coomassie brilliant blue staining to check equal loading.

Polyacrylamide gel (for 2 mini-gels, 10%)

Seperation gel	**Stacking gel**
2.5 ml lower gel 4 X buffer	2.5 ml upper gel 4 X buffer
4.9 ml H_2O_{DEPC}	6.6 ml H_2O_{DEPC}
2.5 ml acrylamide solution 40%	0.8 ml acrylamide solution 40%
50 µl 10% ammonium persulfate	100 µl 10% ammonium persulfate
5 µl TEMED	10 µl TEMED

Note: Ammonium persulfate is always freshly prepared.

10x SDS running buffer (pH 8,3)	**1 X Towbin buffer**
250 mM Tris	25 mM Tris
1.92 M Glycine	192 mM Glycine

1% SDS

Add H_2O_{dest} to 1 Liter

20% Methanol

Add H_2O_{dest} to 1 Liter

1 ×PBS-T buffer

NaCl 8 g

KCl 0.2 g

Na_2HPO_4 1.44 g

KH_2PO_4 2.4 g

0.1% Tween-20

Dilute in 800 ml of distilled H_2O.

Adjust the pH to 7.4 with HCl. Add H_2O to 1 liter. Sterilize by autoclaving.

2.8 Expression of recombinant barley Kaurene Synthase Like (HvKSL)

2.8.1 Hv*KSL* transformation of electro competent cells

An aliquot of electrocompetent *E. coli* BL21 (DE3) pLysS was gently thawn on ice (~15 minutes). 10 ng plasmid DNA was added to the cells and the mixture subsequently transferred to an electroporation cuvette. After electroporation (2.5 KV) 1 ml SOC-medium (see 2.2) was added immediately to the cells. Cells were restored by gently shaking at 37 ℃ for 30-60 minutes, and were plated on LBA-plates (containing the appropriate antibiotics: Chloramphenicol 50 μg/ml and Kanamycin 50 μg/ml) for selection.

2.8.2 SDS-PAGE and Western blot analysis of HvKSL protein expression

20 µg of each protein sample was prepared using 5 X sample loading buffer (Pierce, Rockford, USA). Samples were denatured at 95℃ for 5 minutes and used for electrophoretic separation using acrylamide gels (1 mm, 10%). The stacking gel and separation gel were prepared as described in 2.7.2. The PAGE system was assembled and filled with 1 X SDS running buffer. The denatured samples were loaded on the gel and run at 100 V for 30 minutes and 120 V for 1.5 h until bromophenol blue front reached the end of the gel. After electrophoresis, gels were transferred to 1 X Towbin buffer. For Western blot analysis, nitrocellulose membrane (pore size 0.2 µm, Dassel, Germany) was cut and activated by methanol. Subsequently, the Western sandwich was assembled and put in cold 1 X Towbin buffer. After blotting (0.1 A for 1 h. 1 mA per cm^2 gel area), proteins were transferred to nitrocellulose membrane. The membrane with transferred protein was washed three times with PBST buffer for 10 min each time. Non-specific binding sites were blocked using 5 % (w/v) milk powder (ROTH, Germany) in PBST buffer at 4℃ overnight. After three times washing with PBST buffer, the nitrocellulose membrane was immunostained using 1:500 diluted rabbit anti-His6-tag polyclonal antibody (Dianova, GmbH. Hamburg, Germany) for 1.5 h. Thereafter, the nitrocellulose membrane was washed (5 times, 10 min each) with PBST bufferfollowed by transfer to 1:10000 diluted anti-rabbit-HRP secondary antibody. The detection was done using the ECL Plus Western Blotting Detection Kit for HRP (Amersham Biosciences, www.gelifesciences.com) on a Molecular Dynamics Storm 840 (Cambridge Scientific, www.cambridgescientific.com).

2.8.3 Small and large scale expression of HvKSL protein

For all four gene constructs (Hv*KSL1*, Hv*AK370792*, Hv*ent-CPSL1*, Hv*syn-CPS*), overnight cultures of transformed *E. coli* BL21 (DE3) pLysS from a single colony were prepared and inoculated in 50 ml LB medium (containing chloramphenicol and kanamycin) at a dilution of 1:100. The culture was incubated under shaking at 37 ℃ until OD (600 nm) of 0.6-0.8 was reached. Then 1 ml of samples (for sample preparation see below) was taken and respective OD-values were noted. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM. 1 ml samples were taken 1, 2, 3 and 4 hours after induction and OD-values were noted. The respective samples were then centrifuged at 13000 rpm for 5 minutes to harvest the cell pellet. The cell pellet was dissolved in 50 µl denaturing cell disruption buffer (100 mM NaH_2PO_4, 10 mM Tris/HCl, 8 M urea, pH 8.5) and supplemented with 50 µl protein loading buffer. For SDS-PAGE, 15 µl of each sample was loaded at an OD of 0.5 (less volume for higher OD). For the large scale expression, a single colony was used to inoculate in 20 mL LB medium containing 50 mg/L Kanamycin and Chloramphenicol and allowed to grow overnight at 37 ℃ on a shaker. The overnight culture was used to inoculate 1 l LB with a ratio of 1:100. The bacteria were grown at 37 ℃ under shaking until the OD (600 nm) reached 0.6-0.8. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM. Cells were harvested 4 h after induction by centrifugation at 8000 rpm, 30 minutes, 4 ℃ (Beckmann centrifuge, rotor JA10). 2 X 1 ml samples were taken (centrifuge at 13000 rpm for 5 minutes, discard supernatant) for SDS-PAGE solubility test.

2.8.4 Solubility test of HvKSL protein

Cell pellet was resuspended in 100 µl native buffer (50 mM Tris/HCl, pH 8) and sonicated. Then centrifugation was done at 13000 rpm for 5 minutes. The supernatant (soluble fraction) and the pellet (insoluble fraction) were separated. 50 µl protein loading buffer were added to the supernatant, loading 20 µl for SDS-PAGE. 50 µl denaturing cell disruption buffer (see above) was used to dissolve the pellet, adding 50 µl protein loading buffer and load 10-15 µl for SDS-PAGE. The denatured samples were loaded on the gel and run at 100 V for ~1.5 h until the bromophenol blue front reached the end of the gel. After SDS-PAGE electrophoresis, the gel was stained with the staining buffer for 30 minutes and destained with the destaining buffer for overnight.

Staining buffer	**Destaining buffer**
Coomassie-blue: 0.1%	Ethanol: 10 %
Ethanol: 40 %	Acetic acid: 5 %
Acetic acid: 10 %	

2.8.5 Inclusion body isolation and solubilization of HvKSL protein

The cell pellet from large scale production was resuspended in buffer 1 (see below) at 5 ml/g, 1.5 mg lysozyme was added per gram cell pellet, and the cell suspension was incubated 30 minutes at 4 ℃ . Cells were disrupted by sonication 3 times (5 minutes per time) and then paused on ice to keep the solution at low temperature. To degrade the contamination of genomic DNA, $MgCl_2$ with a final concentration of 3 mM and DNase were added and incubated for 30 minutes at room temperature. 1/2 of total volume of

buffer 2 was added and the suspension was incubated on ice for 30 minutes. The solution was centrifuged by 20 minutes at 4℃, 15000 rpm. The pellet was washed 4 times with buffer 3 and the same centrifugation condition was repeated. Then the pellet containing the insoluble protein fraction was stored at -20℃ until further preparation. For the inclusion body solubilization, 5 ml/g Sol-buffer was added to the inclusion body pellet with DTT at a final concentration of 50 mM. The solution was incubated 2 h at RT. Then the pH value was adjusted to 4 by dropwise addition of acetic acid. The protein solution was dialyzed against to Tris/HCL 100mM, pH 8 by changing buffer 3 for 3 times in 6 h.

Buffer 1	**Buffer 2**
100 mM Tris/HCl	60 mM Na-EDTA
1 mM Na-EDTA	6% Triton x-100
pH 7	1.5 M NaCl

Bufffer 3	**Sol-buffer**
100 mM Tris/HCl	100 mM Tris/HCl
20 mM Na-EDTA	5 mM Na-EDTA
pH 7	6 M GdmCl pH 8.5

2.8.6 HvKSL protein purification

To simplify the purification process and to get useful terpene cyclase enzyme for enzyme assay, the terpene cyclase genes were constructed with an N-terminal His6- tag allowing purification by affinity chromatography using Ni-NTA column material. The

dialyzed protein solution was collected and loaded on an equilibrated Ni-NTA column (HisPrep 5 ml prepacked column by GE Healthcare) at a flow speed of 1 ml/min by the help of a peristaltic pump (P1 pump, Pharmacia Biotech). The flow through fraction was collected. The column was washed with 5 CV running buffer. Then the target protein was eluted from the column with 5 CV elution buffer. Fractions of the washing and elution steps were collected at a size of 1 CV each. From each fraction 20 µL were loaded on 10% sodium dodecyl sulfate-polyacrylamide gels (see chapter 2.7.2). After electrophoresis, gels were stained with staining solution (20% Methanol, 60% H_2O, and 20% colloidal Coomassie blue Roti ®-Blue, Roth, Karlsruhe, Germany) for 1 h followed by overnight destaining in destaining buffer.

Running buffer	**Elution buffer**
100 mM Tris/HCl	100 mM Tris/HCl
pH 8	250 mM Imidazol pH 8

2.9 Preparation of root cell walls

The barley roots were crushed under liquid nitrogen in smaller pieces (0.5 -1.0 cm) and washed in cold distilled water in a glass beaker (ratio of 1:2 in relation to the fresh weight of the roots). The material was filtrated through a small meshed gaze (mull) and squeezed. This washing and filtration step was repeated several times (4-5 washes).

Then the fibers were extracted with a mixture of methanol/chloroform (1:1). To remove the methanol/chloroform mixture the roots were filtrated again. This extraction procedure was repeated 5 - 7 times. Finally the fibers were washed with acetone and dried under sterile conditions in glass Petri dishes. The fibers were crushed with a

mortar and pistil using liquid nitrogen under sterile conditions to receive cell wall powder for further experiments. According to the report of Sposato (1995), the method used here was modified. The cell walls were stored at RT.

2.10 Staining of *F. graminearum* and *P. indica* in root tissues

For visualization of root colonization, hyphae of *F. graminearum and P. indica* were stained with the chitin-specific dye WGA-AF 488 (Molecular Probes, www.invitrogen.com). The roots inoculated with *F. graminearum* and *P. indica* were fixed in TCA (trichloroacetic acid) solution (ethanol: 800 ml, chloroform: 200 ml, TCA: 0.15%) and washed 5 times for 3- 5 minutes in PBS buffer. The roots were transferred to 10 ng /ml WGA-AF-488 dissolved in PBS solution and roots were stained by vacuum infiltration (1.2 kg/cm^2). The roots were transferred to 50% glyceroland prepared for confocal laser-scanning microscopy (CLSM).

2.11 Confocal laser-scanning microscopy (CLSM) and non invasive mGFP5-ER localization

Subcellular localization of mGFP5-ER and WGA-AF 488-stained *F. graminearum* and *P. indica* was performed by CLSM. In both cases, root and fungal cells were excited with a 488 nm laser line to detect emission of mGFP5-ER and WGA-AF 488 at 505–530 nm.

2.12 Database searching, sequence and phylogenetic analysis

The following databases were used for BLAST searches: National Centre for Biotechnology Information (NCBI) at http://www.ncbi.nlm.nih.gov/.

Barley full length cDNA Database, http://barleyflc.dna.affrc.go.jp/hvdb/index.html, developed by the National Institute of Agrobiological Sciences (NIAS) (Matsumoto et al., 2011). The phylogenetic analysis was performed using Molecular Evolutionary Genetics Analysis (MEGA) software, version 5.0 (Tamura et al., 2011). The pair-wise deletion option was adopted on the treatment of amino acid gaps on the terpene cyclase multiple alignment produced by the PILEUP program of the Genetics Computer Group (GCG) Wisconsin package. Phylogenetic trees were obtained from neighbor joining analysis derived from the p-distance method. In the phylogenetic tree construction, the confidence levels assigned at various nodes were determined after 5,000 replications using the Interior Branch test (Sitnikova et al., 1995).

3. Results

3.1 Stable Root Transformation System (STARTS)

3.1.1 Development of a stable root transformation system (STARTS)

We aimed to establish a fast and robust root transformation system for functional characterization of newly expressed proteins in plant roots. In a first experiment, we identified conditions that provided a fast and efficient generation of roots from calli of the barley cultivar Golden Promise. Toward this end, the scutella of immature embryos were isolated from developing kernels of plants at 12-14 days after anthesis and transferred to callus induction medium (Table 2.1) yielding calli by rapid cell division (Figure 3-1B) After two subcultures at an interval of 2 weeks, calli were transferred to root induction medium (Table 2.2) where multiple roots emerged from single callus cells within 2-3 weeks (Figure 3-1D). A comparison of the transformation method for production of transgenic T1 plants with that of stable transgenic root cultures is given in Figure 3-1.

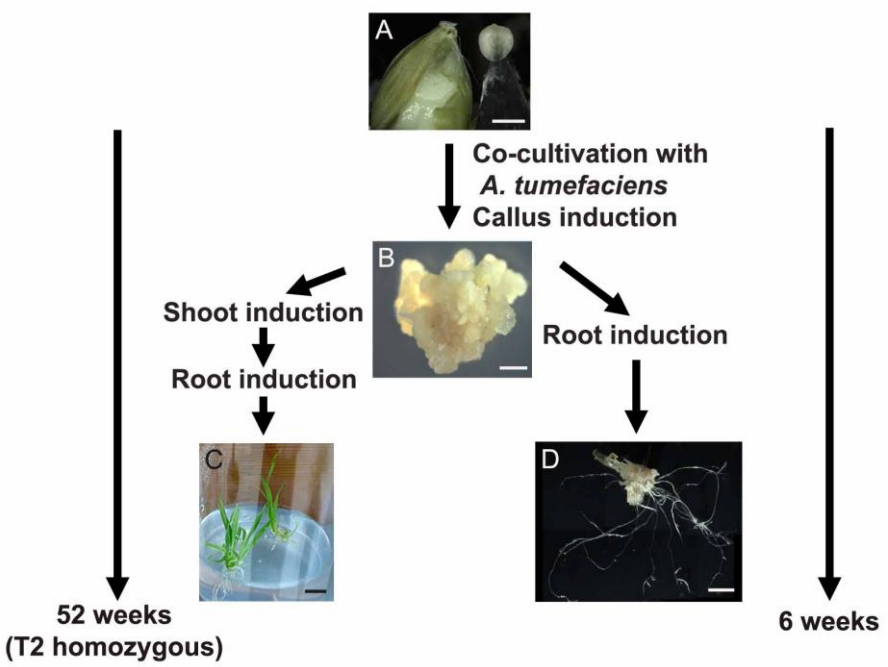


Figure 3-1. Comparison of the conventional transformation method production of T2 homozygous transgenic seedlings and the production of stable transgenic T1 roots with STARTS.

Scutella (*) are isolated from immature embryos without the embryo axis (a) (see Materials and Methods details on the preparation of scutella) and co-cultured with *Agrobacterium tumefaciens* for transformation. Calli are regenerated from transformed scutella cells (b). For (conventional) plant transformation, calli are subjected to shoot and thereafter to root induction medium to obtain T1 plants. For STARTS, calli (b) are subjected to root induction medium to regenerate roots from individual, transformed cells. Transformed roots are obtained within 6 weeks in comparison with the (conventional) shoot transformation procedure by which homozygous root material is obtained after 52 weeks. Notably, each transgenic root regenerated by STARTS represents an independent integration event. The multiplicity of transgenic events and, as result, regenerated roots diminishes the occurrence of integration-associated phenotypes during functional studies. A higher amount of heterozygous roots regenerated from single shoots by shoot first transformation approaches are required to obtain an equally confident result.

Importantly, the medium contained coconut milk and various vitamins. Coconut milk is the liquid endosperm of *Cocos nucifera* and is known as a supplier of growth regulators for improving the development of tissues in culture (Mathias, 1986; Kobayashi, 1995; Neumann, 2009). Coconut milk was prepared from coconuts distributed by regular supermarkets. For transformation, coconut milk was filter sterilized and aliquoted (non-used aliquotes were frozen at -20 ℃). After six weeks, a plenty of roots had developed to analyze the effect of dominant transgenes for transcription and translation. The *Agrobacterium tumefaciens*-based approach relies on the regeneration of roots after shoot induction, which takes about 16 weeks. Although these roots can be

forwarded to functional studies, we observed regeneration of roots from shoot tissue is highly limited. Alternatively, roots of homozygous T1 transformants might be used. However, these roots are only accessible after 12 months. This reliable but time consuming conventional *A. tumefaciens*-based transformation method is currently used for functional genomics in barley. To verify the applicability of our new method for generating stably transformed roots, we also performed *A. tumefaciens*-mediated transformation (see Experimental Procedures for details). For transformation, scutella were isolated from Golden Promise kernels (Figure 3-1A) and co-cultivated for 2 days with *A. tumefaciens* strains AGL1 (Lazo et al., 1991) harbouring the pLH6000-Ubi::*sGFP* construct (Figure 3-2A) to allow transformation of scutella cells with s*GFP* (synthetic green fluorescent protein). *sGFP* was placed under control of the constitutive maize ubiquitin (Ubi) promoter. The binary vector for plant transformation pLH6000 (DNA-Cloning Service, Hamburg, Germany) harbors the selectable marker hygromycin phosphotransferase (hph), which detoxifies the aminocyclitol antibiotic hygromycin C. GFP was microscopically observed in transformed cells at 1-2 days after co-cultivation (Figure 3-2B, C) and in dividing cells at 1-2 weeks after *A. tumefaciens* transformation (Figure 3-2D). GFP was also observed in calli regenerated from transformed scutella cells at 2-3 weeks after co-cultivation (Figure 3-2E). Upon transfer of the calli onto root induction medium (see Materials and Methods), roots were developing from single callus cells within 2-3 weeks (Figure 3-2F, G). Notably, we did not detect chimeric roots containing transformed and non-transformed tissue which is consistent with the expectation that roots emerge from a single cell to develop

root primordia. This experiment suggested that the method, named STARTS (stable root transformation system), is applicable for rapid analysis of protein function in plant roots.

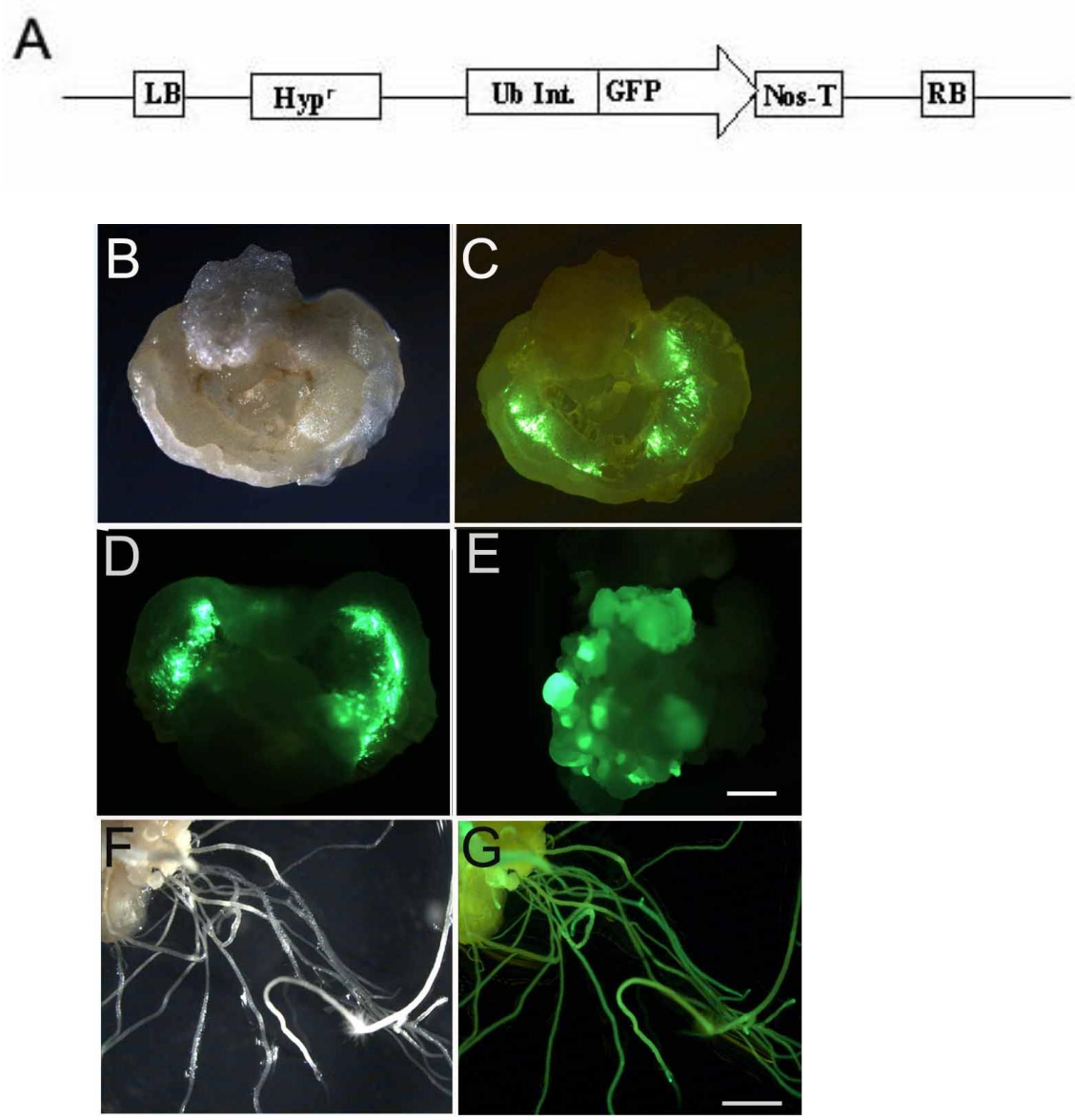


Figure 3-2. Overexpression of synthetic green fluorescent protein (sGFP) in barley roots by STARTS.

(a) Schematic diagram of the construct pLH6000-Ubi::*sGFP* for GFP overexpression using STARTS. (b) Scutellum (without the embryo axis) 2 days after transformation by co-cultivation with *Agrobacterium tumefaciens.* (c) Fluorescence image of (b) showing sGFP expression in cells of the scutellum. (d) Expression of sGFP was observed in dividing scutellum cells at 1–2 weeks after co-cultivation. (e) Callus had been formed from *sGFP*-transformed scutellum cells at 2–3 weeks after co-cultivation. (f, g) sGFP expressing roots regenerated from single sGFP-transformed callus cells. sGFP was visualized by excitation at 450–490 nm and emission was detected at 510–530 nm using a stereofluoresence microscope (Leica, Germany, http://www.leica-microsystems.com). Bars = 2 mm (b–e) and 2 cm (f, g).

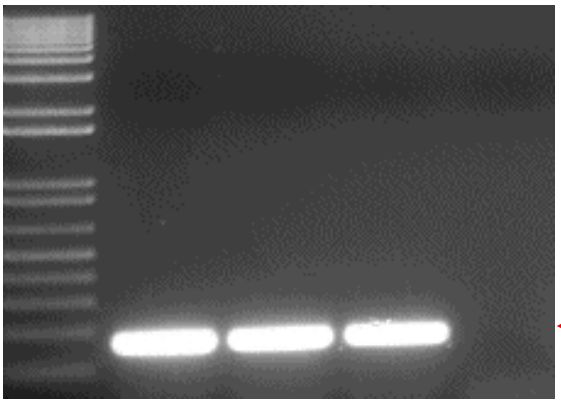
3.2 STARTS is suitable for gene silencing

3.2.1 Silencing of Hv*EXPANSIN B1*

Next, I addressed the question whether STARTS could be applied for gene silencing. Therefore, we monitored RNAi-mediated gene silencing of the endogenous barley Hv*EXPANSIN B1* (Hv*EXPB1*) gene that is required for root hair development (Kwasniewski and Szarejko, 2006). Scutella from immature embryos of cultivar Golden Promise were co-cultured with *A. tumefaciens* strain AGL1 containing the pIPK007-Hv*EXPB1*-RNAi plasmid which harbours a 155 nt sense-antisense orientation fragment of a non-conserved region of Hv*EXPB1*. The cloning of the gene of interest and destination vector pIPK007-RNAi (Himmelbach et al., 2007) used for transformation are shown in Figure 3-3. After *A. tumefaciens* co-cultivation,

regenerated calli were grown for 4 weeks and thereafter transferred to root induction medium. 2 weeks later, the numbers of root hairs were counted in three different root zones: (1) Elongation/maturation zone, (2) maturation zone I, and (3) maturation zone II (Figure 3-4A). Roots generated from Golden Promise scutella transformed with the empty vector (control) developed root hairs (Figure 3-4B, D, F, H). By contrast, Hv*EXPB1*-silenced roots showed a strongly reduced number of root hairs in all root zones (Figure 3-4C, E, G, H). In addition, quantitative real time PCR (qRT-PCR) was performed to determine the degree of Hv*EXPB1* silencing. Consistently, the amount of Hv*EXPB1* transcript was strongly reduced in silenced roots (Figure 3-4I).

A



B

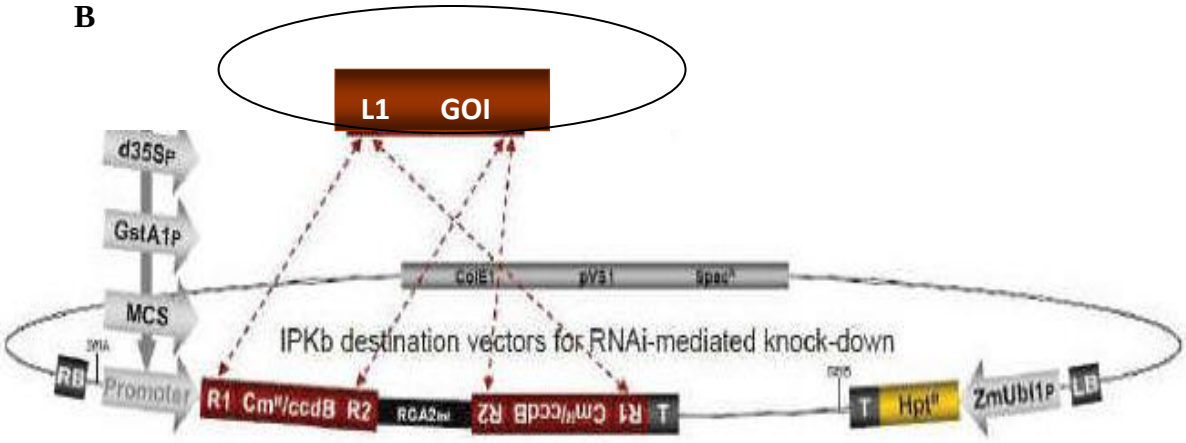
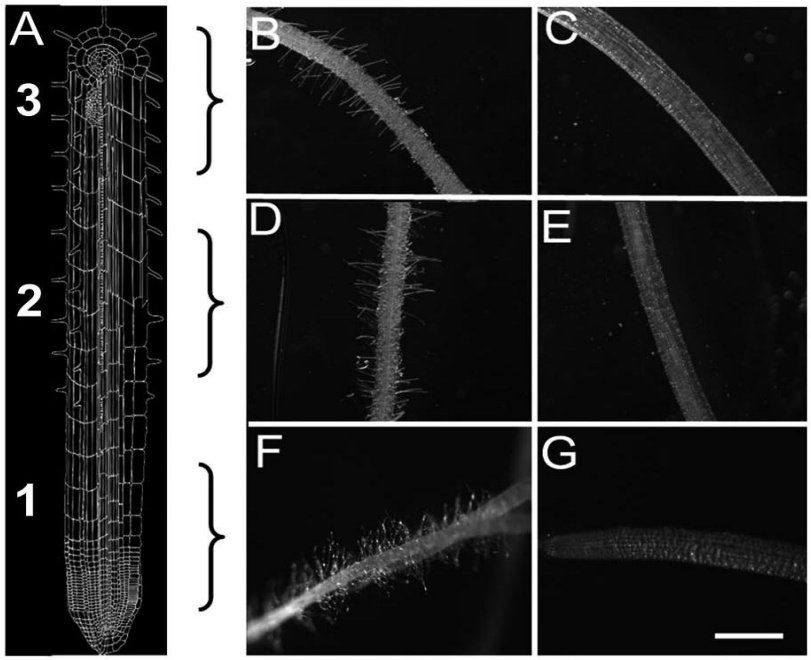


Figure 3-3. Cloning of Hv*EXPANSIN B1* gene into destination vector pIPKb007-RNAi

(a) PCR amplification of 155 bp fragment of Hv*EXPANSIN B1*. M: 1 kb plus DNA ladder, 1-3: 155 bp Hv*EXPANSIN B1* gene fragment, 4: negative control. (b) Schematic representation of binary destination vectors pIPKb007-RNAi vector. The destination cassettes of the RNAi vectors consist of R1 (attR1 recombination attachment site), *Cm*^{r} (chloramphenicol acetyltransferase gene), *ccdB* (negative selection marker), and R2 (attR2 recombination attachment site) sequences. The RNAi vectors further contain the wheat *RGA2* intron (I) separating the inverted repeat of the GATEWAY destination cassettes. Transcription is terminated either by the *A. tumefaciens nos* (t) or the CaMV *35S* termination signal (T). Other components of the vector; i.e. RB (right border), ColE1 (origin of replication for *E. coli*), pVS1 (origin of replication for *A. tumefaciens*), *Spec*^{r} (streptomycin/spectinomycin bacterial resistance), LB (left border), and the plant selection marker *Hpt*^{r} (hygromycin phosphotransferase) controlled by the maize *ZmUbi1* promoter (Himmelbach et al., 2007).



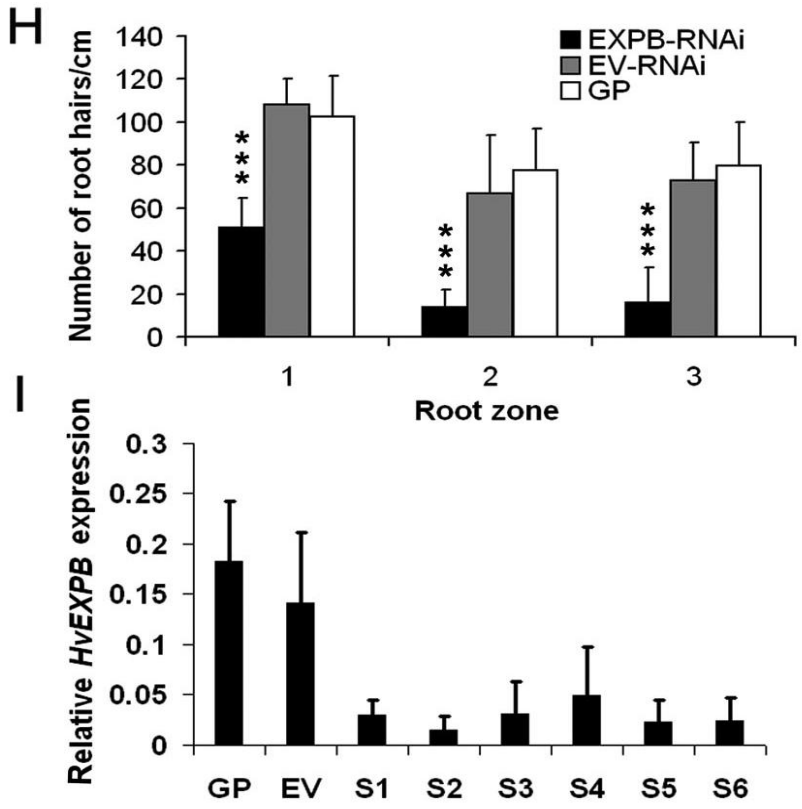


Figure 3-4. Silencing of Hv*EXPANSIN B1* by STARTS.

(a) Root hairless phenotype was observed at the elongation- and meristematic zone (1), maturation zone I (2), and maturation zone II (3) using a binocular microscope. (b, d, f) Root hair phenotype in roots transformed with the empty vector. (c, e, g) Root hair phenotype in roots transformed with the Hv*EXPB1* RNAi construct. Bar = 2 mm. (h) Number of root hairs per cm in root zones (defined in (a)) of roots obtained from wild type Golden Promise (GP), after empty vector transformation (control, EV-RNAi), and after Hv*EXPB1* RNAi (silencing, Hv*EXPB1*-RNAi). Means with standard errors of three independent experiments in which 120 roots were analyzed are displayed. Asterisks indicate significance at P = 1.83E-04 (zone 1), P = 3.40E-03 (zone 2), P = 1.79E-05 (zone 3) using Student's t-test. (i) Amount of Hv*EXPB1* transcript determined by quantitative real-time PCR in GP roots, empty vector transformed roots (EV), and various roots silenced in Hv*EXPB1* (S1–S6). The levels of Hv*EXPB1* transcripts were normalized to Hv*UBIQUITIN*. Displayed are means of three technical replicates with standard deviations.

3.2.2 STARTS is applicable to silence GFP in roots stably overexpressing GFP

Although these analyses proved the potential of STARTS for root gene silencing, we were interested to determine the efficiency and stability of the method. Therefore, a 280 nt *GFP*-RNA interference (RNAi) fragment was selected and put into silencing vector p7U-GFP-RNAi (DNA-Cloning service, www.dna-cloning.com) with an inverted promoter (Figure 3-5A). The transformation vector p7U-GFP-RNAi was confirmed by SfiI digestion and approximately 700 bp including partial GFP fragment and sequence on the vector were cut off from the vector (Figure 3-5B). Transgenic scutella derived from immature T3 Golden Promise embryos stably overexpressing *sGFP* driven by the Cauliflower Mosaic Virus 35S promoter (35S) were co-cultured with *A. tumefaciens* strain AGL1 carrying the 280 nt *GFP*-RNA interference (RNAi) vector p7U-GFP-RNAi. *GFP* silencing was observed in regenerated calli and roots (Figure 3-6A-F). Furthermore, *GFP* silencing was stable and was observed in leaves generated from *GFP*-silenced calli (Figure 3-6G, H).

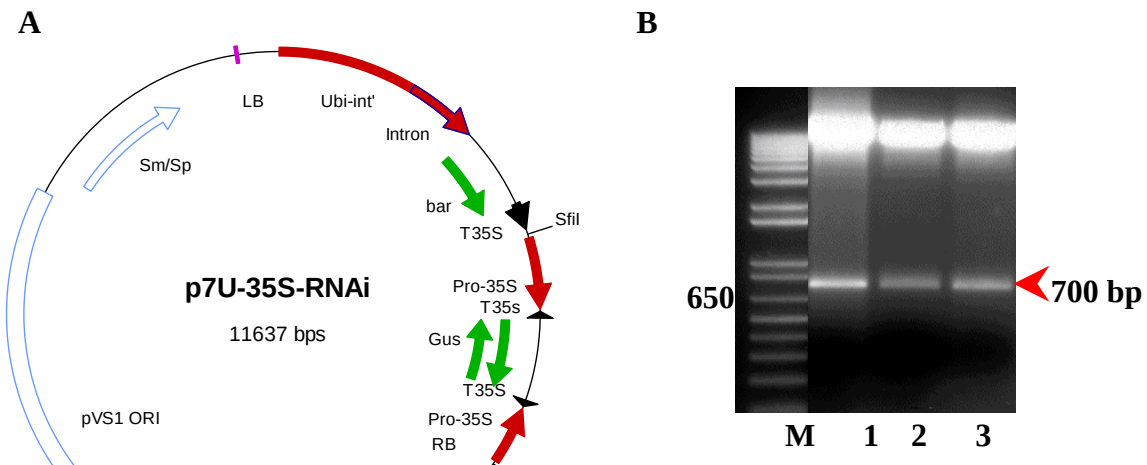


Figure 3-5. Utilization of binary vector p7U-35S-RNAi for gene silencing and confirmation of p7U-35S-GFP-RNAi construct.

(a) Schematic representation of binary destination vector p7U-35S-RNAi vector. Bar*:* *phosphinotricine acyl transferase* gene, Ubi-int: *ubiquitin* gene promoter and its first intron, T35S: 35S terminator, (DNA-Cloning service). (b) *GFP*-RNAi insert confirmation by SfiI digestion in destination vector p7U-35S-RNAi vector. M: 1 kb plus DNA ladder, 1-3: positive clones indicated after digestion.

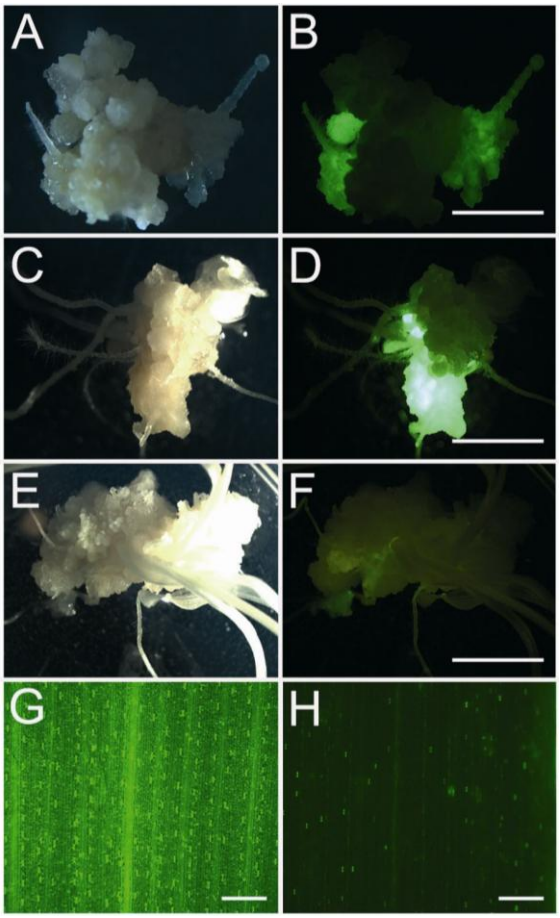


Figure 3-6. Silencing of *GFP* in calli, regenerated roots, and regenerated leaves by STARTS.

(a, b) Partial silencing of *GFP* in a callus. (c, d) *GFP* silencing in roots regenerated from a *GFP*-silenced area of a callus. (e, f) Complete silencing of *GFP* in a callus. (a, c, e) Bright field images. (b, d, f) Fluoresence images showing GFP expression. (g) Leaf of a transgenic plant expressing *GFP* under control of the cauliflower mosaic virus 35S promoter. (h) *GFP* silencing in shoot of T1 progeny regenerated from a *GFP*-silenced callus. GFP was visualized by excitation at 450–490 nm and emission was detected at 510–530 nm using a stereo-fluorescence microscope (Leica, Germany, http://www.leica-microsystems.com). Bars = 2 mm.

As expected, different degrees of silencing were obtained in different transformed calli. However, in 70% of all cases >50% of calli cells were silenced as indicated by the absence of GFP (Figure 3-7A). In addition, the extent of *GFP* silencing in roots generated from *GFP*-silenced calli was determined. About 50% of all roots displayed an intermediate or complete reduction in GFP protein expression (Figure 3-7B). qRT-PCR analyses revealed a drastically reduced level of *GFP* transcripts even in those roots that exhibited an intermediate silencing phenotype (Figure 3-7C). Consistent with the reduced transcript levels in *GFP*-silenced roots, GFP protein accumulation was strongly reduced in all samples (Figure 3-7D). In sum, STARTS was successfully applied to overexpress and silence genes. The data further show that STARTS-mediated gene silencing is stable and transferred to shoots. *GFP* silencing was also observed in plants of the T1 progeny.

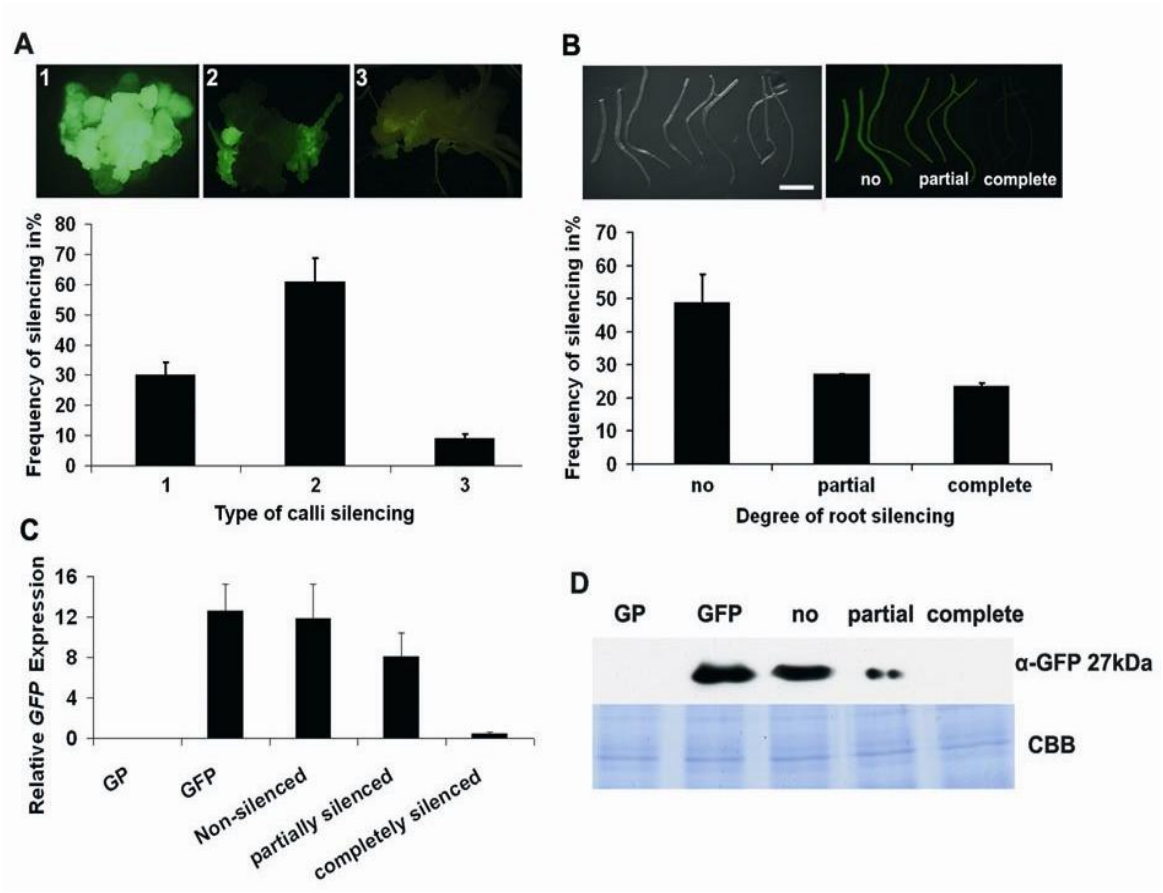


Figure 3-7. *GFP* silencing by STARTS.

Scutella were isolated from immature embryos of the T3 generation of a barley line constitutively overexpressing *GFP*. (a) Degree of *GFP* silencing in calli regenerated from scutella overexpressing *GFP* after co-cultivation with *A. tumefaciens* carrying a *GFP*–RNAi construct. The number of calli not silenced (1), partially silenced (2), or completely silenced (3) was determined. Data represents means of 60 independent calli obtained from two independent experiments. Error bars represent standard deviations. (b) Degree of *GFP* silencing in roots regenerated from calli. Three different silencing phenotypes were distinguished (right panel): None-silenced roots (no), partially silenced roots (partial), and completely silenced roots (complete). For comparison, the respective bright field image is shown (left panel). Data presented are means of 120 independently regenerated roots obtained from two independent experiments. Error bars represent standard deviations. Bar = 2 cm. (c) Degree of *GFP* silencing in roots of Golden Promise (GP), *GFP* overexpressing roots (GFP), and roots displaying various degrees of *GFP* silencing (as described in (b)) using qRT-PCR. (d) Immuno-detection of GFP using monoclonal anti-GFP antibody. The coomassie brilliant

blue (CBB)-stained gel shows equal sample loading. Samples used were the same as described in (c).

3.3 STARTS is suitable for protein localization studies

The subcellular localization of proteins adds significant information to a prediction of their function. In order to investigate the suitability of STARTS for this kind of analysis, we transformed scutella with a modified version of *green fluorescent protein* (*mGFP5-ER*), which is provided with a 5´-terminal signal peptide sequence and a 3´-terminal HDEL sequence to ensure Endoplasmic Reticulum (ER) localization (Haseloff and Siemering, 2005). For our analysis, *mGFP5-ER* was under control of the constitutive 35S promoter. As an independent control, we generated Golden Promise plants that stably expressed *mGFP5-ER.* In roots germinated from seeds of stably transformed *mGFP5-ER* plants (Figure 3-8A, C) as well as in root tissue derived from roots transformed and generated by STARTS (Figure 3-8B, D), the GFP was clearly detected in the ER (Figure 3-8A, B) and in the nuclear periphery (Figure 3-8C, D) by confocal laser-scanning microscopy. These results demonstrated the suitability of STARTS for cell biological protein localization studies.

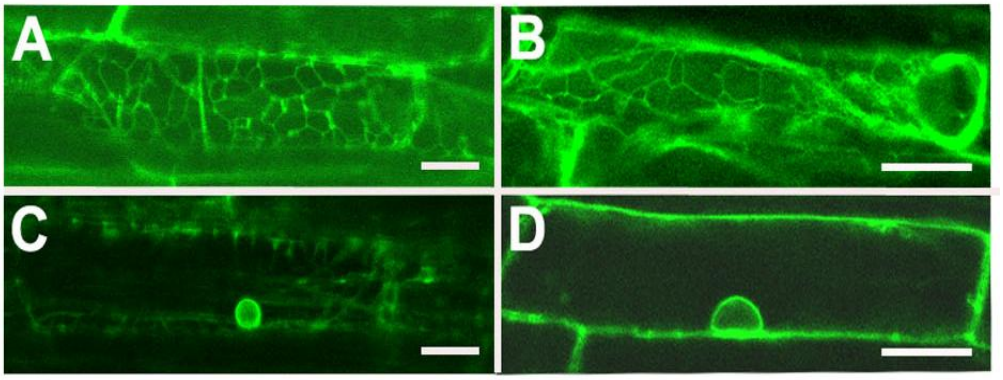


Figure 3-8. Non-invasive mGFP5-ER localization using confocal laser-scanning microscopy.

(a–d) mGFP-ER localization in the ER (a, b) and nuclear periphery (c, d) of roots from stably transformed plants (a, c) and roots transformed by STARTS (b, d). GFP emission (510–530 nm) was detected after excitation with a 488 nm laser line using a TCS-SP2 CLSM (Leica, Germany, http://www.leica-microsystems.com). Bars = 20 µm.

3.4 STARTS is an efficient method to analyze protein function in pathogenic and symbiotic barley root-microbe interactions

We were interested whether STARTS was applicable to study the effect of barley proteins in root-microbe interactions. Therefore, we studied the impact of *BAX INHIBITOR-1*(*BI-1*), a gene exerting cell death inhibitory activity and strong phenotypes in plant interactions with microbes, on two fungi with contrasting life styles, the pathogenic fungus *Fusarium graminearum* and the symbiotic fungus *Piriformospora indica* (Hückelhoven et al., 2003; Deshmukh et al., 2006; Babaeizad et al., 2009). Both microbes follow a cell death-dependent root colonization strategy and were shown to be controlled upon overexpression of *BI-1* in barley plants (Deshmukh et al., 2006; Babaeizad et al., 2009). We transformed scutella with *Agrobacterium* strain AGL1 carrying binary vector pLH6000-Ubi::*GFP-BI-1* under the control of the constitutive maize ubiquitin promoter. Another set of scutella was transformed with pLH6000-Ubi:: *GFP* as control. Roots transformed with these constructs were morphological indistinguishable (Figure 3-9).

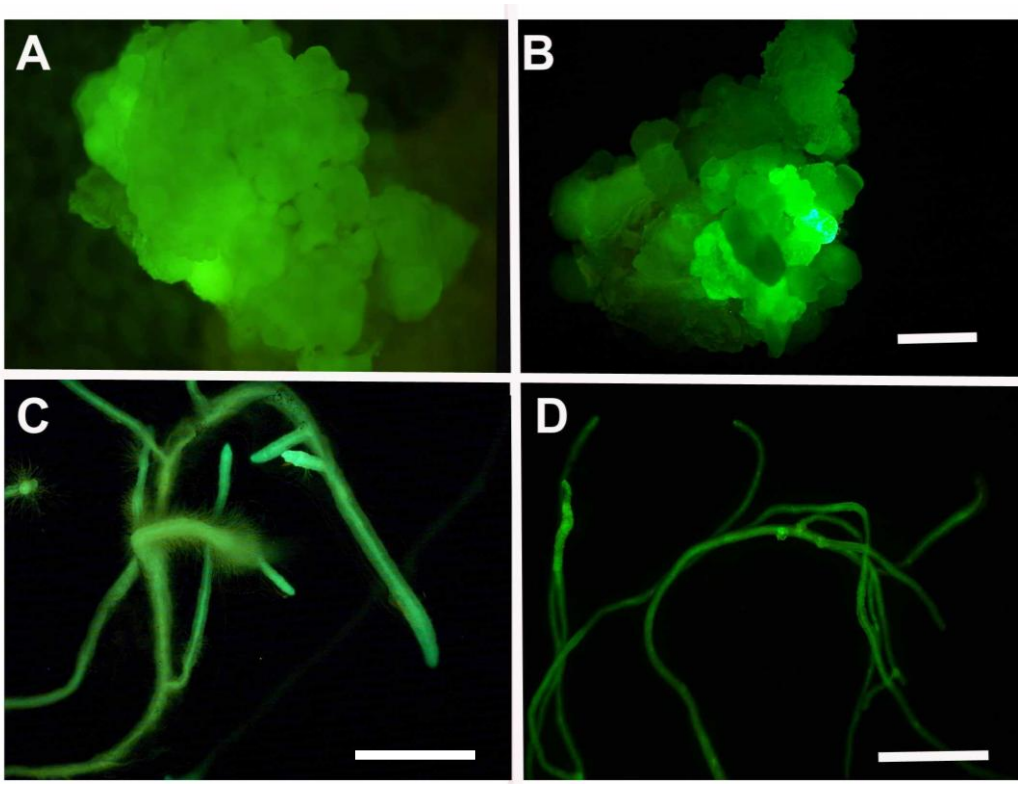
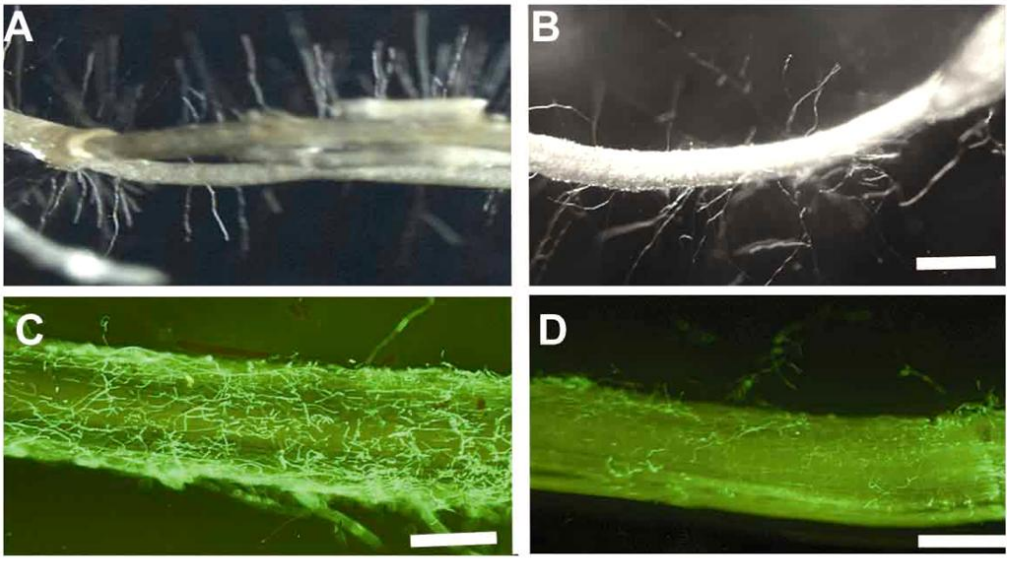


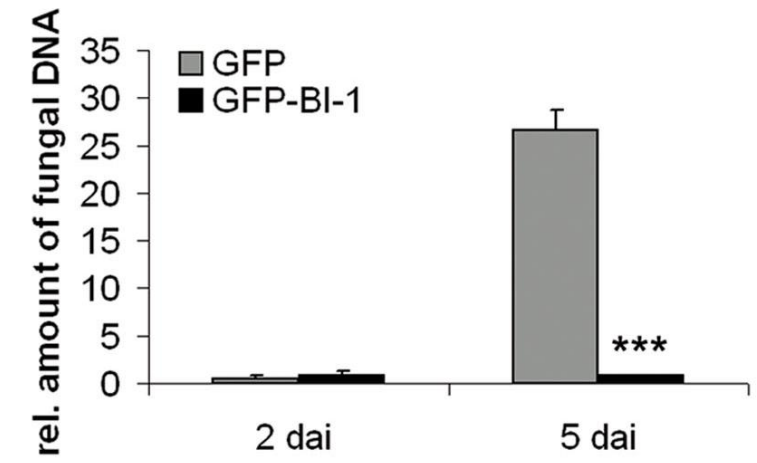
Figure 3-9. Morphology of calli and roots transformed with *GFP* and *GFP-BI-1*.

(a, c) *GFP* expression in barley calli and roots. (b, d) *GFP-BI-1* expression in barley calli and roots. No differences were observed in calli and roots transformed with either *GFP* or *GFP-BI-1*. GFP was visualized by excitation at 450–490 nm and emission was detected at 510–530 nm using a stereo-fluorescence microscope (Leica, Germany, http://www.leica-microsystems.com). Bars = 6 mm (a-b), 1 cm (c) and 2 cm (d).

Subsequently, roots were inoculated with conidia of either *F. graminearum* or *P. indica* and harvested at 2 and 5 or 3 and 7 days after inoculation (dai), respectively. For visualization of root colonization, hyphae of *F. graminearum* were stained with the chitin-specific dye WGA-AF 488 (Molecular Probes, www.invitrogen.com). Microscopy observation showed that root colonization was reduced obviously in the *GFP-BI-1* expressing roots as compared to *GFP* expressing roots (Figure 3-10C, D). In addition, the infected roots were subjected to qRT-PCR for the quantification of fungal DNA as indicator for fungal colonization. *F. graminearum* (Figure3-10E) as well as *P. indica* (Figure3-10F) showed a significantly reduced colonization rate in roots overexpressing *GFP-BI-1*. These results strongly suggest that the newly developed STARTS method can be efficiently applied to study protein function in root-microbe interactions in crop plants such as barley.



E


rel. amount of fungal DNA
35
30
25
20
15
10
5
0
GFP
GFP-BI-1
***
2 dai
5 dai

F

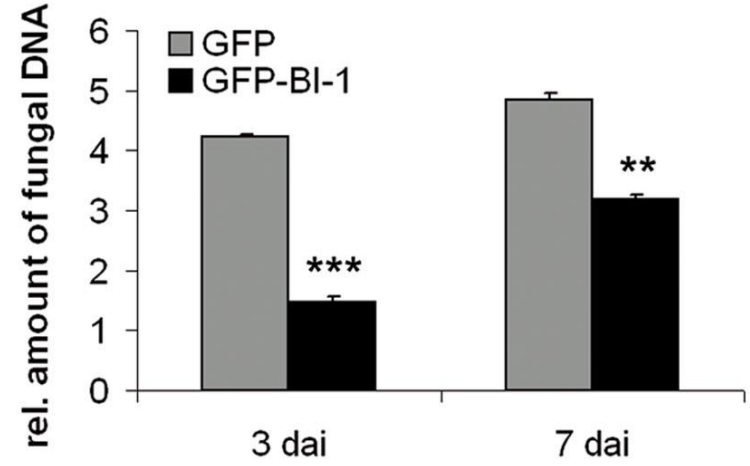

rel. amount of fungal DNA
6
5
4
3
2
1
0
GFP
GFP-BI-1
***
**
3 dai
7 dai

Figure 3-10. Hv*BI-1* affects barley root colonization by pathogenic and symbiotic fungi as indicated by STARTS.

(a–d) Visualization of root colonization by *F. graminearum* in barley roots overexpressing *GFP* and *GFP* fused to Hv*BAX INHIBITOR-1* (*GFP-BI-1*). Hyphae of *F. graminearum* were stained with the chitin-specific dye WGA-AF 488 (Molecular Probes, www.invitrogen.com). (a, c) *GFP* overexpressing roots colonized by *F. graminearium* at 5 dai. (b, d) *GFP-BI-1* overexpressing roots colonized by *F. graminearium* at 5 dai, respectively. Root colonization was obviously reduced in the *GFP-BI-1* expressing roots as compared to GFP expressing roots at 5 dai as indicated in c and d. Scale bars = 1 cm (a-b) and 1,5 mm (c-d) (e) *Fusarium graminearum* infection in barley roots overexpressing *GFP* fused *BAX INHIBITOR-1* (*GFP-BI-1*) as compared with roots overexpressing *GFP* (GFP). Colonization was determined in barley roots at 2 and 5 dai by quantitative real-time PCR using primers specific for *F. graminearum TUBULIN* (Fg*TUB*) and for barley *UBIQUITIN* (Hv*UBI*). Root colonization was significantly reduced in the *GFP-BI-1* expressing roots as compared to *GFP* expressing roots as indicated by Student's t-test (P = 1.4E)02 [5 dai]). Displayed are means with standard errors of two independent biological experiments. (f) *Piriformospora indica* colonization in barley roots overexpressing *GFP-BI-1* as compared to roots overexpressing *GFP* (GFP) at 3 and 7 dai. Colonization was determined in barley roots by quantitative real-time PCR using primers specific for *P. indica INTERNAL TRANSCRIBED SPACER* (Pi*ITS*) and for barley *UBIQUITIN* (Hv*UBI*). Root colonization was reduced significantly in the GFP-BI-1 expressing roots as compared to GFP expressing roots as indicated by Student's t-test (P = 1.65E)03 [3 dai], P = 4.56E)03 [7 dai]). Means with standard errors of three independent biological experiments are displayed.

3.5 Sequence mining and alignment of putative barley kaurene synthase genes

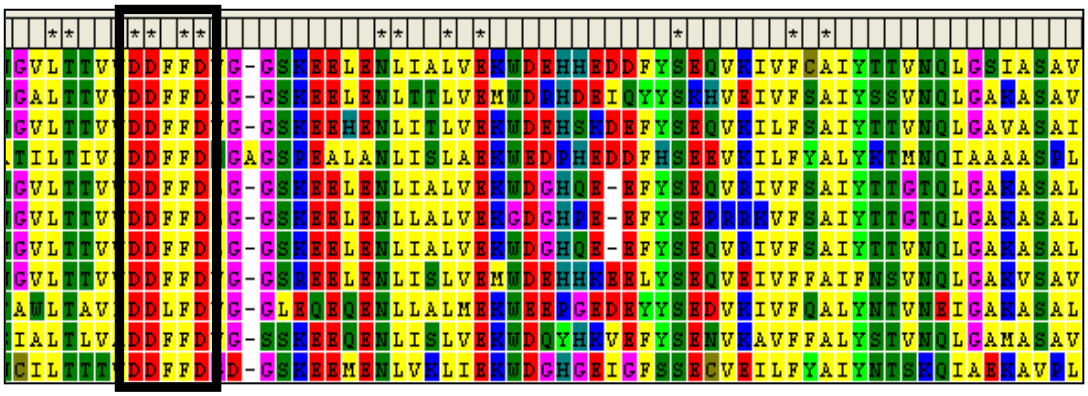
Kaurene synthase-like genes are a huge gene family and presumably involved in diterpenoid and hormone biosynthesis in plants (Otomo et al., 2004). The possibly homologous ESTs for most of the kaurene synthase (*KS*) genes in rice, barley other plant species were presented by Margis-Pinheiro et al.(2005). Accordingly, the barley kaurene synthase-like genes (Hv*KSL*) correlation to rice Os*KS1A* to Os*KS6* gene sequences were mined and summarized in my work (Table 3.1) by using data available at http://barleyflc.dna.affrc.go.jp/hvdb/index.html. Based on the ExPASy protein analysis, it was revealed that some accession numbers, for instance, Y551436 and AK362274. Both encoding the Hv*KSL1* genes, were found to stand for the same gene. I further compared sequence similarities of *KS-like* genes between barley and rice. Notably, the similarity between Hv*KSL1* (AY551436) and Os*KS1a* is approximately 78%, while Hv*KS2* (AK248484) and Os*KS2* as well as Hv*KS4* (AK251219) and Os*KS4* show a sequence similaritiy of 72% and 61%, respectively. Nevertheless, Hv*KS-like* genes most probably have orthologs in rice as suggested by the presence of similarities in the database.

Table 3.1. **Sequence similiraities between barley (Hv*KSL*) and rice (*OsKSL*).**

	GenBank acc.No (Full length gene)	Similarities to rice (bp)
Barley EST	**Hv*KSL1***	**Os*KS1A* (AY347876)**
bu973186(bi949839)	AY551436 AK362274 same gene	78%
	AK362172	69%
Barley EST	**Hv*KS2***	**Os*KS2* (AY347879)**
bu980524	AK354668 AK355281same gene	70%
ca029634	AK248484	72%
Barley EST	**Hv*KS3***	**Os*KS3* (AB089272)**
ca016270	AK362437 AK372766 same gene	58%
ca028666	AK373561	60%
ca017814 ca028301 ca003053 al507968	AK249885	66%
bu998927	AK371248 AK251025 same gene	49%
Barley EST	**Hv*KS4***	**Os*KS4* (AY347880)**
ca026265 ca028353 ca020167	AK251219 AK354443 AK355937 AK355849 same gene	61%
Barley EST	**Hv*KS5***	**Os*KS5* (AY347881)**
ca023387	AK366293 AK367564 same gene	58%
	AK357068 AK373421 AK376328 AK356311 U49482 same gene	59%
bq467634(bu983945)	AK373804 AK248251 AK358505 same gene	61%
bu970070	AK334748(Wheat)	
Barley EST	**Hv*KS6***	**Os*KS6* (AY347882)**
ca023387	AK366293 AK367564 same gene	43%
bu978833	AK373807 AK373621 AK371181 AK360767 same gene	49%

3.6 Phylogenetic analysis of kaurene synthase genes in barley

Previous work reported that the Hv*ent-KS1a* and Hv*KS-like 4* genes in barley were upregulated at 3 and 7 dai by *P. indica* (Schäfer et al., 2009). By blasting the recently published barley cDNA database, I found that the two ESTs belong to one coding sequence with the accession number Hv*AK370792* in the NCBI database. In addition, blast results indicated that Hv*AK370792* shared high similarities to Os*KS4* (88%). In order to determine if the barley genome contains additional *KS-like* genes, blast searches were performed continually by using Hv*AK370792* as query. In total, 17 KS-like proteins were found to share high similarities in barley and rice (Figure 3-11). Sequence alignment of putative barley and rice *kaurene synthase* (*KS)* genes was produced by the MEGA5 program (Tamura et al., 2011) and the results showed that all genes contained the aspartate-rich domain DDFFD, which is supposed to function as a divalent metal ion-diphosphate complex binding site in terpene cyclases (Yamaguchi et al., 1996). Furthermore, these sequences also include small stretches of identical amino acids in the N-terminal region close to the SAYDTAW motif, which has been proved to be essential for terpene cyclase activity (Kawaide et al., 2000).



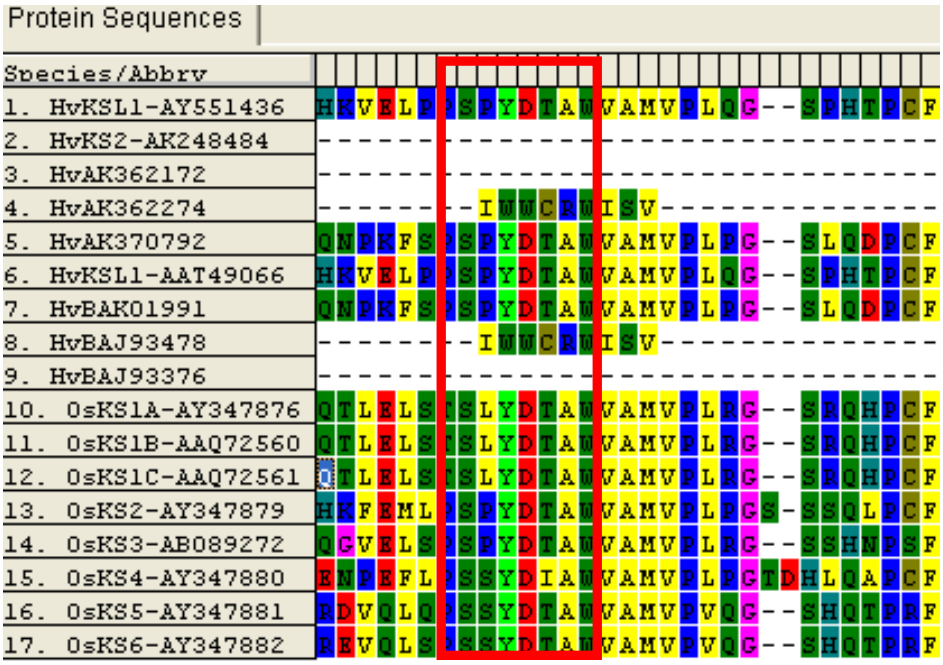


Figure 3-11. Sequence alignment of putative barley and rice *kaurene synthase* (*KS*) genes. The alignment was produced using the MEGA5 program. Gaps have been introduced to maximize alignment and conserved amino acids were highlighted in different colors. The conserved aspartate-rich domain DDXXD and SAYDTAW motif were boxed in black and red, respectively.

Based on selection of the conserved parts by MEGA5, a phylogenetic unrooted tree was generated for the putative KS-like proteins of rice and barley (Figure 3-12). The analysis showed that HvKSL1 protein is clustered with another barley KS-like protein (AK362172) and evolved separately from the OsKS1 protein. HvAK370792 is clustered with OsKS4 in a separate subgroup. Additionally, HvKS2 shares high evolutionary relationship with OsKS2. No similarity was found for OsKS5 or OsKS6 protein in barley.

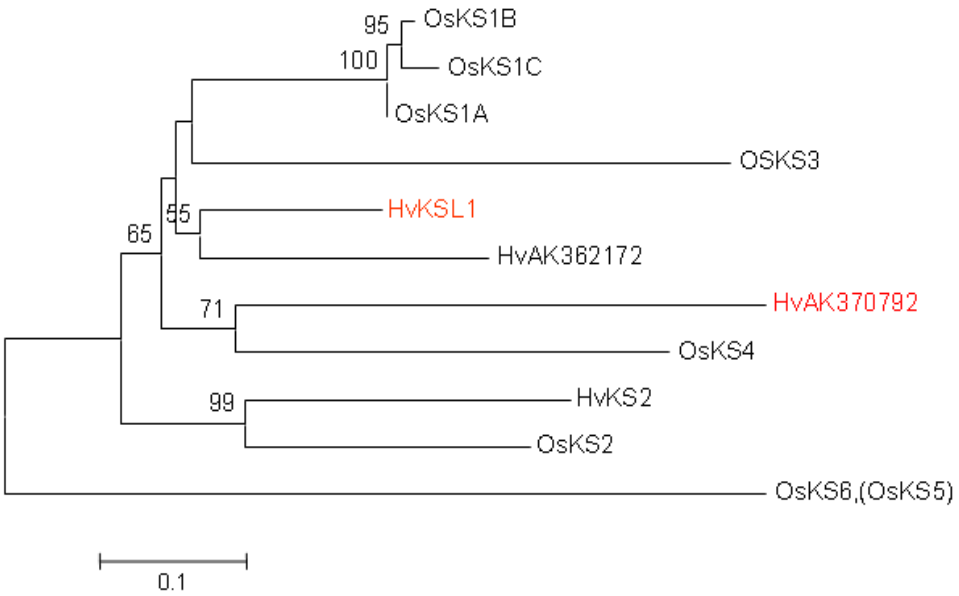


Figure 3-12. Phylogenetic unrooted tree of barley and rice kaurene synthase genes.

The tree was generated from a ClustalW multi-alignment using the Molecular Evolution Genetic Analysis (MEGA5) software with neighbor-joining, p-distance, pairwise deletion, and 5,000 bootstrap replications. The following kaurene synthase like proteins were used and sequences were obtained from NCBI database. For rice: OsKS1A (AY347876), OsKS1B (AY347877), OsKS1C (AY347878), OsKS2 (AY347879), OsKS3 (AB089272), OsKS4 (AY347880), OsKS5 (AY347881), and OsKS6 (AY347882). For barley: HvKSL1 (ay551435), HvAK370792 (AK370792), HvKS2 (AK248484), and HvKS4 (AK355849).

3.7 Prediction of chloroplast transit peptide (cTP) for the terpene cyclase

As mentioned above (Figure1-6), the copalyl diphosphate synthase (CPS) and the kaurene synthase (KS) are the branch point for GA and phytoalexin biosynthesis in plant plastids (Otomo et al., 2004). Geranyl geranyl diphosphate (GGDP) is

converted by the ent- respectively syn- copalyl diphosphate synthase into the stereoisomers ent- resp. syn- copalyl diphosphate (ent-CDP) or (syn-CDP) (Otomo et al., 2004). Previous report demonstrated that two distinct ent-CDP synthases (OsCyc2 and OsCPS1) and a syn-CDP synthase (OsCyc1) catalyze the first step of rice diterpene biosynthesis. Moreover, OsCPS1 was proven to be involved in the GA biosynthesis, while OsCyc2 was involved in the phytoalexins biosynthesis (Otomo et al., 2004). By using the rice *syn-CPS* (Os*Cyc1*, Accession no.AB066270) (Otomo et al., 2004) gene as query AK364238 was identified having 75% similarity to Os*syn-CPS* and named Hv*syn-CPS* (Figure 3-13). The protein sequences of Hvent-CPSL1 (Spielmeyer et al., 2004), Hvsyn-CPS, HvKSL1 (Spielmeyer et al., 2004), HvKS2 and HvKS4 (identified in my work, listed in the Table 3.2) were analyzed with the ChloroP program (http://www.cbs.dtu.dk/services/ChloroP; Emanuelsson et al., 1999) for the presence of plastid transit peptides (Table 3.2). ChloroP uses a neural network method to identify chloroplast transit peptides and cleavage sites based on a training set of proteins with known subcellular localizations. A transit peptide score of greater than 0.5 predicts a chloroplast transit peptide (cTP). For proteins with a predicted cTP, a cleavage site and cleavage site score are also generated. The more positive cleavage site score indicates the higher possibility of a cleavage site. The prediction indicated that all genes, except the Hv*syn-CPS*, contained a plastid transit peptide, which is in coincidence with the previous description that the terprenoids produced by the MEP pathway are biosynthesized in plant plastids.

Accession	Description	Max score	Total score	Query coverage	E value	Max ident	Links
AK364238.1	Hordeum vulgare subsp. vulgare mRNA for predicted protein, complete cds, clone: NIASHv2022P14	4702	4702	100%	0.0	100%	
AB439589.1	Triticum aestivum TaCPS2 mRNA for ent-copalyl diphosphate synthase, complete cds	3319	3319	89%	0.0	93%	U G
AY530101.1	Oryza sativa (indica cultivar-group) syn-copalyl diphosphate synthase mRNA, complete cds	865	865	78%	0.0	75%	U
NM_001058706.1	Oryza sativa Japonica Group Os04g0178300 (Os04g0178300) mRNA, complete cds	843	843	78%	0.0	75%	U G
AK100631.1	Oryza sativa Japonica Group cDNA clone:J023109E08, full insert sequence	837	837	78%	0.0	75%	U E
AB066270.1	Oryza sativa Japonica Group OsCyc1 mRNA for syn-CDP synthase, complete cds	817	817	78%	0.0	74%	U
AK121319.1	Oryza sativa Japonica Group cDNA clone:J023113P09, full insert sequence	593	593	52%	2e-165	75%	U

Figure 3-13. Mining of barley syn-copalyl diphosphate synthase (Hv*syn-CPS*) gene by using Os*Cyc1*.

Both Os*Cyc1* (AB066270) and Hv*syn-CPS* (AK364238) are boxed in red. Acc. Numbers are given in accordance to the NCBI database.

Table 3.2. Summary on the presence of chloroplast transit peptides (cTP) in various terpene cyclases

Protein	Genbank accession no.	Amino acids	cTP score	Prediction*	CS score	cTP length
Hvent-CPS	AY551435	826	0.508	Y	7.586	21
Hvsyn-CPS	AK364238	757	0.452	N	4.498	-

HvAK370792	AK370792	834	0.560	Y	1.932	59
HvKSL1	AY551436	850	0.568	Y	5.637	45
HvKS2	AK248484	631	0.571	Y	2.526	46
HvKS4	AK355849	245	0.501	Y	2.635	33

*** Y, Yes. N, No.**

3.8 *P. indica* affects regulation of Hv*KS-like* genes

To identify whether the expression of kaurene synthase genes were affected by *Piriformospora indica* inoculation, barley roots were inoculated with *P. indica* at 0, 1, 3, 7 days after infection (dai). In three independent experiments, my studies indicated that the Hv*AK370792* gene was upregulated at 3 and 7 dai compared to control treatment (Figure 3-14A). Additionally, the qPCR results revealed that the Hv*KSL1* gene was also upregulated at 3 dai (Figure 3-14C). Moreover, the Hv*KS4* gene showed upregulation at 1 and 3 dai (Figure 3-14B). Taken together, the partial barley *KS-like* genes including Hv*AK370792*, Hv*KSL1* and Hv*KS4* were up-regulated during *P. indica* colonization.

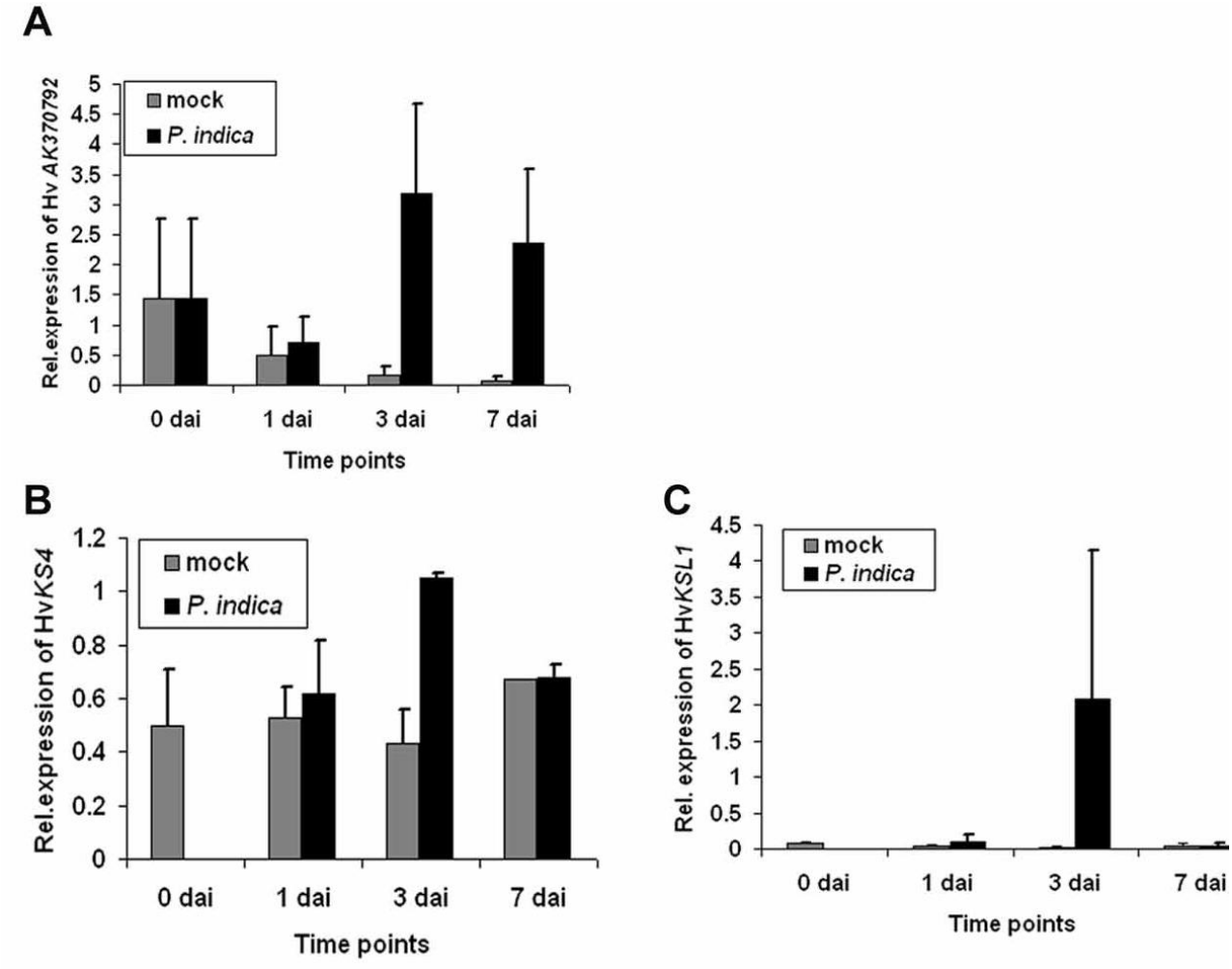


Figure 3-14. *Piriformospora indica* induces various kaurene synthase genes in barley roots.

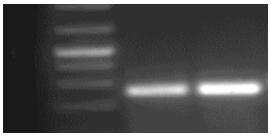
Piriformospora indica colonization of barley roots of cv. Golden Promise as compared to mock-treated roots. (a) Hv*AK370792* was upregulated at 3 and 7 dai. (b) Hv*KS4* gene transcription level was increased at 1 and 3 dai. (c) Hv*KSL1* gene expression was enhanced at 3 dai. The amount of Hv*AK370792,* Hv*KS4* and Hv*KSL1* gene transcripts were determined in barley roots at 0, 1, 3 and 7 dai by quantitative real time PCR. The levels of Hv*AK370792*, Hv*KS4* and Hv*KSL1* gene transcripts were normalized to Hv*UBIQUITIN*. Displayed values are means with standard errors of three independent biological experiments.

3.9 Silencing of Hv*KS-like* genes affects *P. indica* colonization

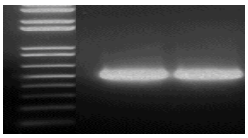
3.9.1 Silencing vector construction

Since the Hv*KS-like* genes were upregulated by *P. indica*, we were interested to know whether silencing of the respective Hv*KS-like* could affect *P. indica* colonization. For this, short fragments of Hv*KSL1* (242 bp) and Hv*AK370792* (300 bp) (Figure 3- 15 A, B) were cloned into p7i-Ubi-RNAi silencing vector by using of the *Hin*dIII and *Bam*HI restriction site (Figure 3-15C). (DNA-Cloning service Hamburg, Germany) under control of an inverted promoter. The marker gene phosphinotricine acyl transferase driven by 35S promoter was used for positive selection of transgenic plants.

A



B



C

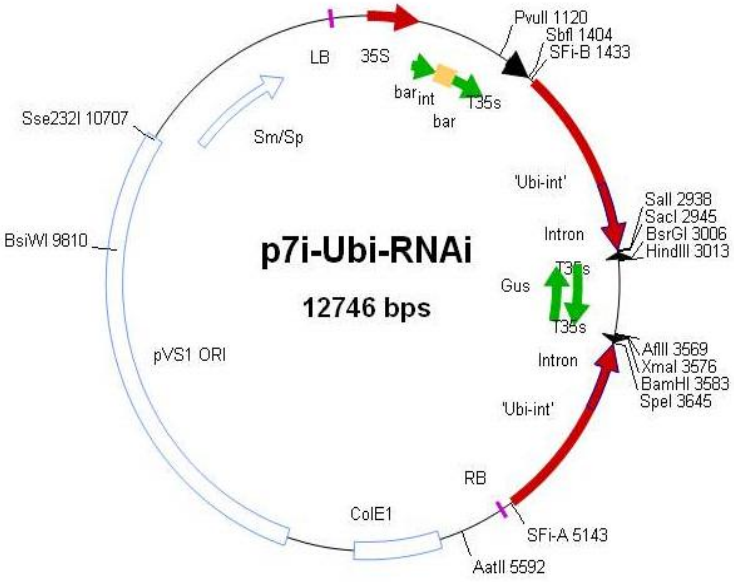


Figure 3-15. Hv*KS-like* partial genes cloning and p7i-Ubi-RNAi vector construction.

(a) PCR amplification of HvKS-like-RNAi fragments. M: 1 kb plus DNA ladder. 1-2: Hv*KSL1* (242 bp), (b) PCR amplification of Hv*AK370792* (338 bp) fragment. M, 1 kb plus DNA ladder. (c) Schematic presentation of p7i-Ubi-RNAi vector. Bar*: phosphinotricine acyl transferase* gene, Ubi-int: ubiquitin gene promoter and its first intron, T35S: 35S terminator, (DNA-Cloning service, www.dna-cloning.com).

3.9.2 Analysis of *P. indica* colonization in Hv*KS* silenced roots by using STARTS

Previous studies revealed that the Os*KSL1* gene is involved in the GA biosynthesis in rice (Otomo et al., 2004), I am interested that whether Hv*KSL1* is also involved in the GA biosynthesis in barley. Furthermore, in order to determine whether silencing of *KS-like* gene will affect *P. indica* colonization, STARTS was applied for the Hv*KS-like*–RNAi roots generation. For each experiment, 100 immature embryos were isolated and transformed with the empty vector (EV), Hv*AK370792*-RNAi or Hv*KSL1*-RNAi. The regenerated root numbers were calculated based on the STARTS. The roots number in Hv*AK370792*-RNAi and Hv*KSL1*-RNAi roots was approximately 700 and 600, respectively, whereas the roots number in EV was approximately 950. Therefore, the root induction ratio was reduced approximately 25-30% compared to the EV control (Figure 3-16A). Subsequently, Hv*AK370792* and the Hv*KSL1*-RNAi silencing roots were inoculated with *P. indica* and harvested at 3 and 7 dai. The qRT-PCR results indicated the tendency of reduced colonization of *P. indica* both in Hv*AK370792RNAi* and *KSL1*-RNAi (Figure 3-16B) although these differences were not significant. Taken together, Hv*AK370792* and Hv*KSL1* genes

might play a role in callus and root generation. Moreover, Hv*AK37079* and Hv*KSL1* genes are required for *P. indica* colonization (Figure 3-16C).

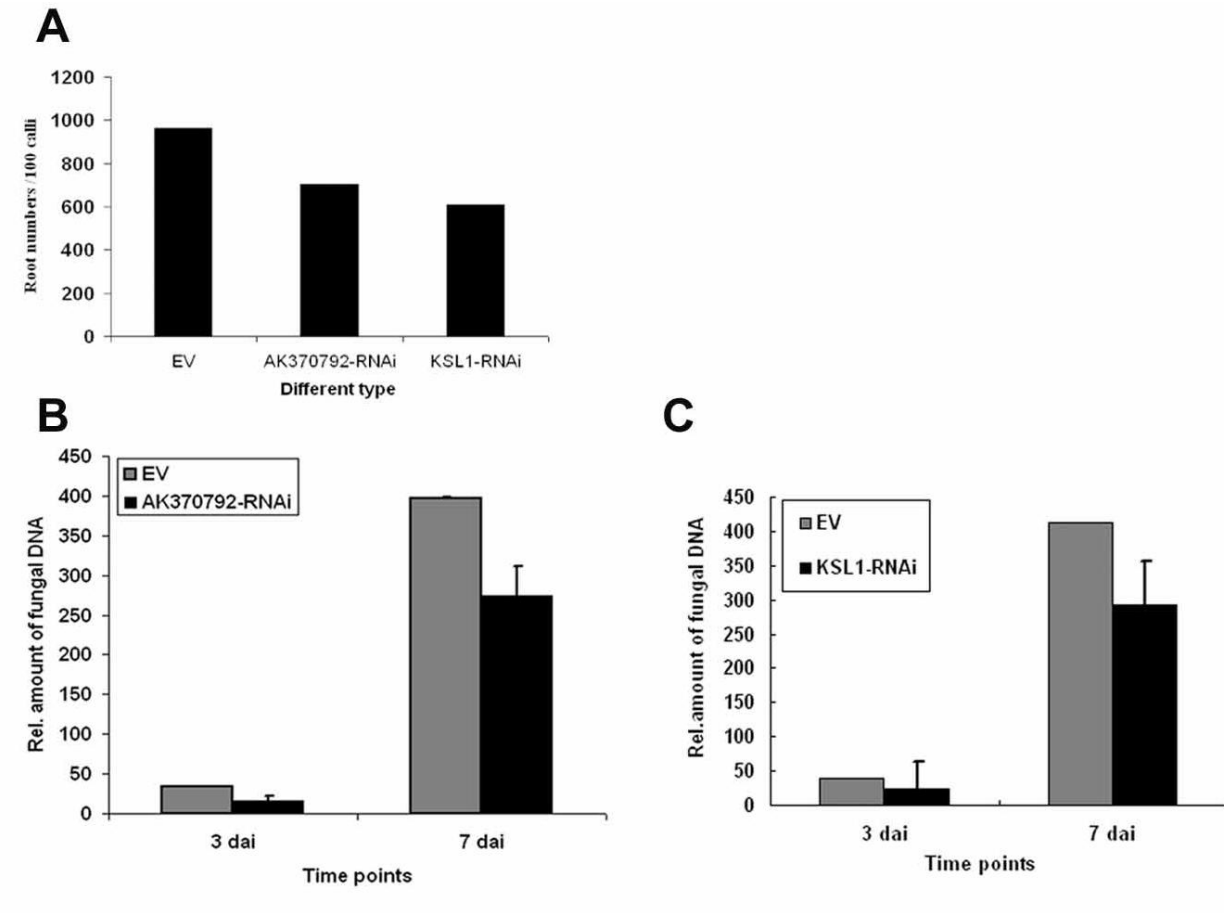


Figure 3-16. Silencing of Hv*KS-like* genes reduced *Piriformospora indica* colonization.

(a) Root regeneration from calli transformed with Hv*AK370792*-RNAi or Hv*KSL1*-RNAi. 100 immature embryos were isolated and regenerated roots were counted. empty vector (EV), Hv*AK370792*-RNAi, Hv*KSL1*-RNAi. (b) *Piriformospora. indica* colonization of barley roots transformed with Hv*AK370792*-RNAi as compared to roots transformed with EV. (c). *Piriformospora. indica* colonization of barley roots transformed with Hv*KSL1*-RNAi as compared to roots transformed with EV. Colonization was determined at 3 and 7 dai by qRT-PCR using primers specific for *P. indica INTERNAL TRANSCRIBED SPACER* (Pi*ITS*)

and for barley *UBIQUITIN* (Hv*UBI*). Displayed are means with standard errors of three independent experiments.

3.10. Generation of barley plants stably expressing Hv*AK370792* and Hv*KSL1*-RNAi constructs

Os*KSL1* was previously reported to be involved in the GA synthesis (Otomo et al., 2004); Hv*KSL1* shared high similarities to Os*KSL1*which might explain the impaired regeneration of roots from calli cells expressing HvKSL1-RNAi (Figure 3-16A). In order to analyze whether silencing of Hv*AK370792* also affect plant development, transgenic plants were generated from calli silenced in Hv*AK370792* or Hv*KSL1*(Figure 3-17B, C) Expression of the silencing constructs was confirmed by semi-quantitative PCR using primers specific for the *phosphinotricine acyl transferase* (*Bar*) gene, which provides glufosinate-ammonium resistance (Figure 3-17A). Silencing efficiency was identified by qRT- PCR (Figure 3-17D-E). The transcription levels of Hv*AK370792*and Hv*KSL1* were reduced in leaves of the two transgenic lines compared to the untransformed plants regenerated from callus. The leaves of Hv*AK370792*-RNAi and Hv*KSL1*-RNAi plants were less dark green compared to the control. Additionally, Hv*AK370792*- and Hv*KSL1*-RNAi lines showed a slower plant development as compared to control plants. These phenotypes are in an agreement with the previous report that the OsKS1-RNAi plants showed dwarf or semi-dwarf phenotypes (Pinheiro et al., 2005).

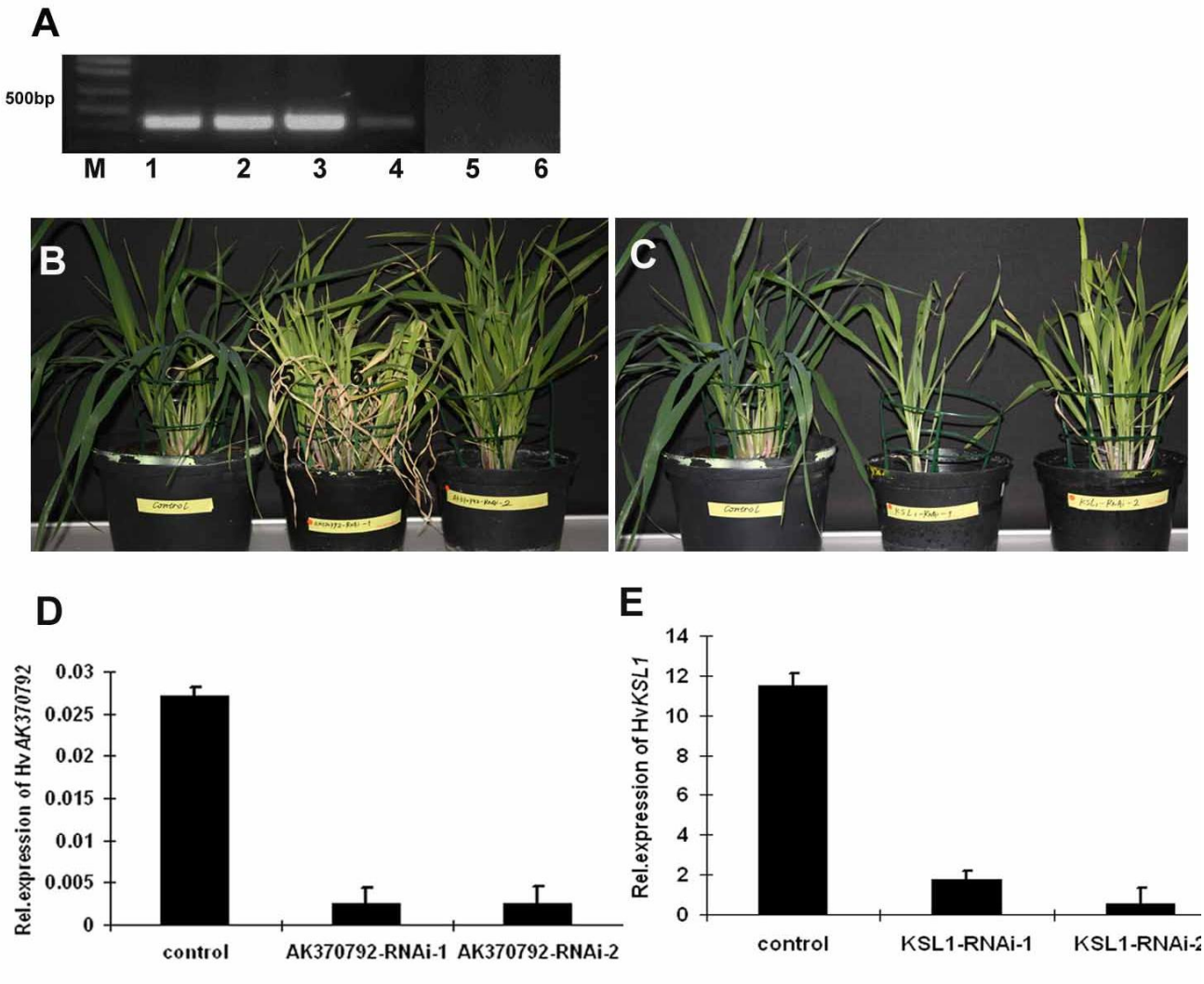


Figure 3-17. Phenotype of Hv*AK370792*-RNAi and Hv*KSL1*-RNAi plants.

(a) Transgenic plants were identified by the amplification of the marker gene *B*ar using semi-quantitative PCR. M: 1 kb plus DNA Ladder, 1-2: DNA samples of Hv*AK370792*-RNAi plants, 3-4: DNA samples of Hv*KSL1*-RNAi plants**.** 5: DNA sample of empty vector-expressing plants. 6: H_2O-$_{DEPC}$ served as negative control (PCR). (b) left: untransformed regenerated plants, middle and right: heterozygous Hv*AK370792* RNAi plants**.** (c) left: untransformed regenerated plants, middle and right: heterozygous Hv*KSL1*-RNAi plants**.** (d-e) Amount of Hv*AK370792* and Hv*KSL1* transcript in leaves determined by qRT-PCR in transgenic Hv*AK370792*-RNAi lines (d) Hv*KSL1*-RNAi lines (e) as compared to

control plants. The levels of Hv*AK370792* and Hv*KSL1* transcripts were normalized to Hv*UBIQUITIN*. Results shown are means ±SD of three technical replicates of QPCR data.

3.11 *In vitro* expression of barley terpene cyclases

3.11.1 Cloning of terpene cyclase genes and expression vector construction

In order to identify the metabolite produced by HvAK370792protein, Hv*KSL1* (2421 bp) and Hv*AK370792* (2322 bp) lacking their putative chloroplast transit peptide were cloned into the prokaryotic expression vector pET-30a (+) to generate recombinant protein. The KS-like enzymes utilize copalyl diphosphate as substrate to produce ent-kaurene, ent-cassa-diene,ent-stemarene or other yet to be identified metabolites(Otomo et al., 2004). The idea was to add the precursor metabolite to recombinant HvKSL1 and HvAK370792 to identify the end products synthesized by both proteins *in vitro*. However, copalyl diphosphate is not commercially available. Therefore, I cloned syn- and ent-copalyl diphosphate synthase (CPS) into pET-30a (+) to produce respective recombinant proteins. I decided to clone both CPS as it is unclear whether HvAK370792 uses syn- or ent-copalyl diphosphate as substrate. Coding sequences of Hv*ent-CPSL1* (2418 bp) and Hv*syn-CPS* (2274 bp) lacking the sequence of the putative chloroplast transit peptides were amplified (Figure 3-18A) and cloned into pET30a (+) (Figure 3-18B).

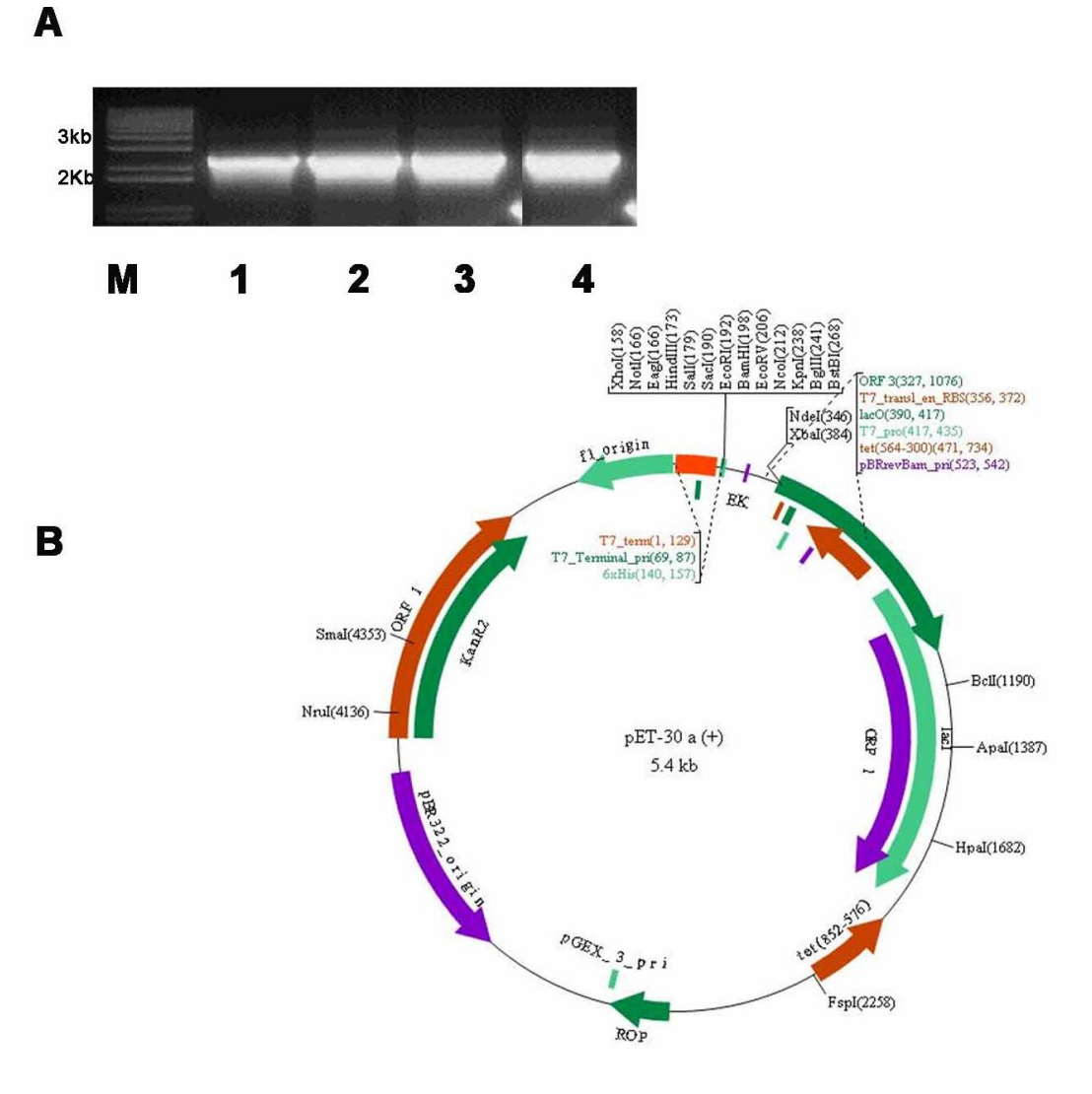


Figure 3-18. Molecular cloning of Hv*AK370792*, Hv*KSL1*, Hv*ent-CPSL1* and Hv*syn-CPS* full length genes.

(a) PCR amplification of the terpene cyclase genes. M: 1 kb plus DNA ladder|, 1: Hv*AK370792* (2322 bp), 2: Hv*KSL1* (2421 bp), 3: Hv*ent-CPSL1* (2418 bp). 4: Hv*syn-CPS* (2274 bp) (b). pET30a (+) vector map.

3.11.2 Expression and purification of recombinant HvKS-like protein

My "*in vitro*" strategy allowed the usage of commercially available geranyl geranyl diphosphate (GGDP) as substrate for recombinant syn-/ent-CPS to produce syn-/ent-CDP, which in turn should serve as substrate for HvAK370792 and HvKSL1 to produce our metabolite/s of interest (Figure 3-19). This metabolite analysis requires expression of the active and purified enzymes in *E. coli*. The final products will be identified by high performance liquid Chromatography (HPLC) combined with mass spectrometry (MS).

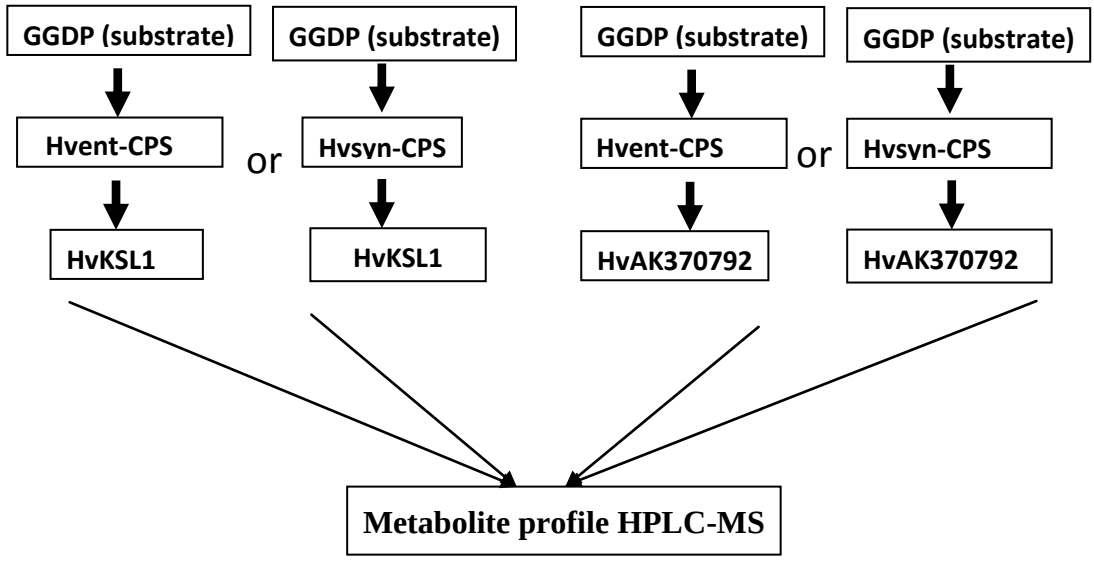


Figure 3-19. The strategy to identify substrates produced by terpene cyclases *in vitro*.

The exact protein sizes of terpene synthases without the chloroplast transit peptide are: Hvent-CPSL1 (91592 Da), HvKSL1 (90963 Da), Hvsyn-CPS (86587 Da) and HvAK370792 (87912 Da); as analyzed by EXPASY-protparam (http://web.expasy.org/protparam/). The purified protein of Hvent-CPS, Hvsyn-CPS, HvKSL1, HvAK370792 will be applied to convert the substrate GGDP to the metabolite/s of interest.

The respective constructs were transferred into *E. coli* BL21 (DE3) plysS for heterologous expression and recombinant protein production. SDS-PAGE analysis showed that protein expressions were induced by 1 mM IPTG at 4 hours after induction (hai) (Figure 3-20A). Furthermore, protein expression was confirmed by Western blot using the anti-His6 polyclonal antibody (Dianova, GmbH Hamburg, Germany). Two conditions (room temperature and 37℃) were used for the induction of protein synthesis. However, the results showed that only HvAK370792 protein expression was detectable (Figure 3-20B, C red box). The immunoreactive bands detected by the anti-His6-tag antibody that could not be assigned to the target protein may be due to protein degradation as well as unspecific crossreaction with histidin-rich host cell proteins. All the four constructs together with the empty vector specifically detected a 35 kDa protein recognized by the anti-His6 polyclonal antibody. By contrast to protein expresssion at room temperature, the 37℃ condition was more suitable for protein expression as indicated by the reduced presenceof unspecific bands. Those unspecific bands probably indicated a stress response, since *E. coli* was grown at room temperature. HvAK370792 protein was purified by Ni-NTA column under denaturating conditions. After loading the sample onto a Ni-NTA column, the target fusion protein HvAK370792 was eluted from the column with elution buffer containing 250 mM imidazole. The purity of the HvAK370792 protein was analyzed by SDS-PAGE (Figure13-20D). The results indicated that not all fusion peptides could be bound to the matrix (some were removed from the

column by the primary salt washing), but large amounts of protein were eluted with 250 mM imidazole elution buffer. To improve the purity of the protein solution a size exclusion chromatography step was performed. Finally the purified fusion protein HvAK370792 was obtained and used for further *in vitro* enzyme assays.

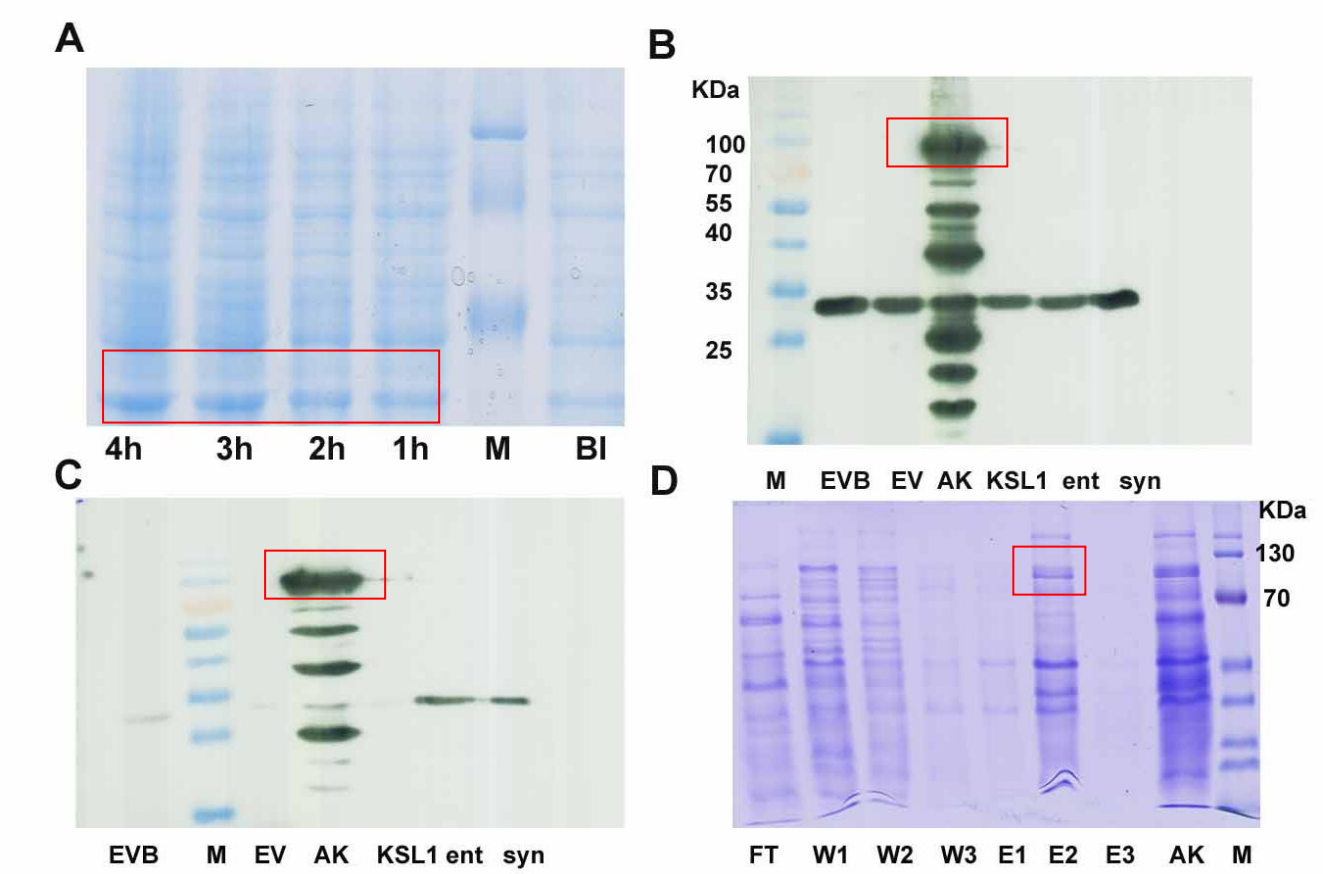


Figure 3-20 Analysis of the terpene cyclase protein induction and expression *in vitro*.

(a) Protein induction was tested at 1 h, 2 h, 3 h, 4 h after induction (hai). 4 hai was identified as optimal time for protein accumulation at high level. BI: Before IPTG induction. The final concentration of IPTG was 1mM. (b) Protein expression under room temperature. HvAK370792 protein expression was detected in immunoblot analysis (boxed in red). EVB: *E. coli* BL21 (DE3) plysS transformed with pET30a (+) empty vector and before IPTG induction, EV: pET30a (+) empty vector induced by IPTG. AK, KSL1, ent, syn: *E. coli* BL21 (DE3)

plysS transferred with Hv*AK370792*, Hv*KSL1* Hv*ent-CPSL1*, and Hv*syn-CPS*, respectively. (c) KS-like protein expression at 37 ℃. Sample names are same as in b. (d) HvAK370792 protein purification was performed by Ni-NTA column. The correct size of HvAK370792 appeared in the washing buffer (w1, w2) and elution buffer (E2) (highlighted in red box). For all experiments, 20 µg total protein per sample was separated by 10 % SDS-PAGE. Immunoblot analyses were performed with anti-His6 polyclonal antibody at 1:5000 dilution.

4. Discussion

4.1 Conventional genetic transformation systems

Plant roots serve as the central organ for anchoring the plant into the soil, and supplying plants with water and salts as well as other nutrients (White and Brown，2010). In other respect, roots are also subjected to abiotic stress including low nitrogen supply, drought, salinity, and heavy metal toxicity (Witcombe et al.，2008) as well as biotic stress caused by microbial pathogens and pests (Cook, 2006; FAO, 2009; Fuller, 2009). In order to better understand the function of genes in plants and root-environmental interactions, plenty of studies have been performed by using the stable and transient transformation methods. In agriculture systems, stable genetic transformation for the production of transgenic barley plants is developed (Tingay et al., 1997; Babaeizad et al., 2009;). However, the generation of transgenic barley plants is time-consuming and laborious and thus too inefficient for high-throughput functionally examinations of proteins. Therefore, transient systems have been developed for barley leaves (Schweizer et al., 1999; Schweizer et al., 2000) but a corresponding system was not available for barley roots. Exiting transient root transformation approaches including virus-based transformation or *Agrobacterium*-mediated gene transformation. Tobacco Rattle Virus (TRV)-based transient gene transformation of roots of *Nicotiana benthamiana* had been established (MacFarlane and Popovich, 2000) but can not be transferred to barley due to virus host specificities. In addition, those systems bear certain disadvantages. For instance, foreign gene of interest should be restricted to a certain size according to the

virus vector requirement. In addition, compared to the stable transformation system, the leaves infected with the virus is more vulnerable to store the foreign protein. Moreover, *Agrobacterium rhizogenes*-mediated gene transfer is mostly not achieved in all cells of a root resulting in patchy transformation genotypes (Limpens et al., 2004). In addition, progenies of these transformed plants show morphologically abnormal traits (Newell, 2000). Therefore, great efforts are required for the efficient method establishment for barley protein functional characterization.

4.2 Establishment of a Stable Root Transformation System (STARTS) for barley

We describe here the establishment of a stable root transformation system for barley, which we called STARTS. We demonstrate that STARTS has certain advantages compared to the above mentioned systems. One of the most voluble characters is the fast generation of large amounts of stable transgenic roots which could pre-screen the effect of candidate genes in a given characters in short time. As an *Agrobacterium tumefaciens*-based transformation technique, scutella of immature embryos are transformed by co-cultivation with the bacteria. The transfer of obtained scutella to selection media allows generation of calli. The novelty of the approach is the transfer of these calli to a root inducing medium (Table 2. 2) to generate uniformly transformed roots. As a principle of the method, regenerated roots originate from one callus cell and thus yield in roots in which all cells have an identical genotype. Most importantly, STARTS accelerate functional studies in roots since continued cell division on root induction medium allows the production of large amounts of roots for the analysis of

proteins in about 6 weeks (Figure 3-1). This time course outcompetes conventional approaches such as the use of roots regenerated after shoot induction, which would be accessible in about 16 weeks and would limit the availability of root tissue for functional studies. In addition the use of roots from homozygous transformants would delay functional studies since the generation would require 8-12 months depending on the transformation procedure used (androgenetic pollen, immature embryos). Importantly, roots produced by STARTS originate from independent transformation and, thus, DNA integration events. Since phenotypes observed in our studies are based on the analysis of dozens of transgenic roots, this greatly enhances the robustness of STARTS for functional analyses as DNA integration-based phenotypes are being reduced.

4.3 Applicability of STARTS

4.3.1 STARTS is a valuable tool for the functional characterization of proteins

Interestingly, a root hairless phenotype was obtained by silencing of Hv*EXPANSIN B1* (Figure 3-4). Moreover, I observed variation in the efficiency of silencing (Figure 3-4I). Notably, I observed silencing of *GFP* under control of the constitutive 35S promoter by STARTS indicating the capability of the system to suppress genes, whose expression is under constitutive regulation (Figure 3-6). I was able to perform non-invasive protein localization studies using roots overexpressing *GFP* equipped with a signal peptide and an endoplasmic reticulum retention sequence (*mGFP5-ER*) (Haseloff and Siemering 2005) (Figure 3-8A-D). These cell and molecular biological analyses indicate that

STARTS represents a powerful tool for the identification and functional characterization of plant factors influencing root organization, architecture, development, cell differentiation, root-leaf communication, as well as nutrient and water uptake. Particularly, nutrient (e.g. phosphorus) and water availability in soil represents limiting factors in modern crop production can be studied with STARTS. It is further considered that global climate changes will elevate these constricts in future crop adaptation (FAO, 2009; Gilbert, 2009; Smit et al., 2009). Current strategies are focused on supplying plants with more efficient root systems thereby optimizing water uptake (White and Kirkegaard, 2010). In this field major achievements have been made in model plants such as *Arabidopsis*. Moreover, a multiplicity of omics information provided by post-genomics era, such as transcriptome, proteome or metabolome data, requires elucidation of their biological significance. STARTS might facilitate the translation of these findings to barley and other recalcitrant plant species and help to improve respective traits in plant roots.

4.3.2 STARTS is suitable for the functional study of proteins in root-micro interactions

Support is given by the interaction of plant roots with mutualistic microbes, such as mycorrhizal fungi or N-fixing bacteria, which considerably improve nutrient and water uptake of roots and enhance plant growth. Importantly, plants are also protected against abiotic and biotic stresses (Oldroyd, 2008; Parniske, 2008; Sherameti et al., 2008). Mutualistic root-colonizaing microbes, such as the fungus *Piriformospora indica*, have been shown to enhance nutrient and water recruitment and to increase abiotic stress

tolerance of plants (Waller et al., 2005; Sherameti et al., 2008; Schäfer and Kogel, 2009; Yadav et al., 2010). The potential of these mutualistic symbioses might be accessible if we will understand the molecular basis of these interactions. For instance, which plant factors stop root pathogen invasion and which processes support mutualistic root symbioses. STARTS might accelerate the identification of respective traits (proteins) and the timely introduction into crop plants. Recent studies reported that stable overexpression of *BAX INHIBITOR-1* (*BI-1*) in barley plants exhibited enhanced resistance to the pathogenic fungus *Fusarium graminearum* as well as *Piriformospora indica* (*P. indica*) (Deshmukh et al., 2006; Babaeizad et al., 2009). BI-1 is an evolutionary conserved, endoplasmic reticulum (ER)-resident protein, which potentially regulates PCD in all eukaryotes (Watanabe and Lam, 2009). Studies indicated that execution of apoptosis as one type of programmed cell death in animals is irreversibly triggered by cytochrome c release from mitochondria via pores formed by BAX proteins (Xu et al., 2008). This type of programmed cell death can be prevented by the expression of BI-1 as it protects cells from the effects of BAX (Sanchez et al., 2000). Genetic transformation of *BI-1* gene will substantially contribute to a better understanding of mechanism underlying resistance to hemibiotrophic pathogens in monotyledonous plants, and to provide critical clues to curb crop diseases caused by *Fusarium* species. As a proof of concept, I confirmed the reduced susceptibility of STARTS-generated roots overexpresssing *BI-1* to both microbes (Figure 3-8.E, F). However, the molecular bases of root colonization and of

the beneficial effects are only partly understood. Much more information for the root-micro interaction would be deciphered based on STARTS.

4.4 Conclusions of STARTS

One inherent drawback of STARTS might be seen in the unpredictability of inserted gene copy numbers in single cells from which roots are regenerated. Another putative disadvantage is that the regenerated roots are not genetically homogenous because each emerging root is regenerated from one embryogenic cell. However, the system allows the simultaneous and fast generation of dozens of roots originating from independent transformation events. Therefore, protein-specific phenotypes should be consistently detectable in almost all roots thereby even reducing the occurrence of misinterpretations or transformation artifacts due to genome insertion effects. My data suggest that STARTS allows to pre-screen the effect of candidate genes in a given trait and to reject ineffective candidates at an early stage. Importantly, calli can be immediately transferred to respective media to regenerate stably transformed plants in which observed phenotypes can be independently confirmed. In sum, STARTS is suggested to elucidate the effect and function of proteins in root development, stress tolerance/resistance or other aspects. With the fast development of the next generation sequencing technology, more plant genomes will be sequenced in near future. Efficient determination of the function of identified genes/proteins on a large scale is a major challenge to improve the productivity and quality traits of crop plants. In addition, roots are the organs most subject to beneficial and microbial interactions; STARTS might provide an efficient method for analyzing the function of proteins in

mutualistic and pathogenic interactions. The applicability of the system for functional studies is further indicated by the second part work of identification of MEP-derived metabolites during barley root colonization by *Piriformospora indica.*

4.5 Genes involved in Gibberellic Acid (GA) biosynthesis in barley

GAs comprises a large family of tetracyclic diterpenoid phytohormones that take part in the regulation of plant growth and development processes. GAs work as endogenous regulator in plant growth and development including seed germination, stem and leaf elongation, flower induction, fruit and seed development in plants (Hedden and Phillips 2000; Davies, 2004; Zhang et al., 2007). Lot of work and time had been spent in the past years in the identification and analysis of components participating in either GA biosynthesis or signaling. Almost all genes encoding the seven GA biosynthesis enzymes (*ent-CPS, ent-KS, ent-KO, ent-KAO, GA20ox, GA3ox, and GA20x*) have been cloned from a number of plant species and different mutants isolated (Hedden and Phillips, 2000; Sakamoto et al., 2004; Yamaguchi, 2008). In rice, 29 candidate genes for seven GA metabolic enzymes were identified by *in silico* screening of the rice genome and cDNA database (Sakamoto et al., 2004). Four *CPS-like*, nine *KS-lik*e, and five *KO-like* genes were identified in the rice genome, while the Arabidopsis genome contains only one gene encoding of CPS (Sun and Kamiya, 1994), KS (Yamaguchi et al., 1998a) or KO Helliwell et al., 1998). Although several *CPS-like* and *KS-like* genes were characterized in rice, only Os*CPS1* and Os*KS1* are involved in GA biosynthesis. Some of the *CPS-like* and *KS-like* genes might encode enzymes involved in phytoalexin biosynthesis. With the exception of

mutants lacking GA2ox, the typical mutant phenotype is dwarfism, a symptom that can be recovered by application of GA (Sakamoto et al., 2004). Dwarf cultivars have certain advantages like more resistant to strong wind, which correlates with increased yields and other important agronomic features. In rice, the isolation and analysis of genes involved in GA biosynthesis are well advanced and a good understanding of their functions has been established. Here we attempt to explore and enhance our understanding of GA metabolism in barley roots prepared by STARTS. The major purpose of this work was to understand the function of barley genes encoding kaurene synthases in the interaction with *P. indica* and GA metabolism.

4.5.1 Terpene cyclase genes: key branch enzymes in the biosynthesis of gibberellins and other secondary metabolites

The classical mevalonic acid (MVA) pathway in eukaryotes and archaea, as well as the pyruvate: C-methyl-D-erythritol-4-phosphate (MEP) pathway in bacteria and plants are the two pathways providing the isoprenoid precursors isopentenyl diphosphate (IPP) and dimethylallyl diphosphates (DMAPP) (Roberts, 2007). In higher plants, IPP and DMAPP are converted to kaurene by the enzymes geranylgeranyl diphosphate synthase (GGPS), ent-copalyl diphosphate synthase (ent-CPS) and ent-kaurene synthase (ent-KS). In rice, the biosynthetic pathway of diterpene phytoalexins has been shown to branch from geranylgeranyl diphosphate and ent-copalyl diphosphate (Otomo et al., 2004). The same authors also discovered that the *Oryza sativa oscps* and *osks* mutants lead to severe dwarf phenotypes without flower and seed development. It is reported that five *KS-like* genes (Os*KS1*, Os*KS2*,

Os*KS3*, Os*KS4*, Os*KS6*) expression level are variable in different organs such as roots, shoots and panicles; in addition, the transcripts level of the five *KS-like* genes mutants (*osks1, osks2, osks3, osks4, osks5*) were lower compared to the normal plants (Pinheiro et al., 2005). For instance, Os*KS1* was expressed predominantly in panicles, and was also highly expressed in stem and leaves. Os*KS2* and Os*KS4* were highly expressed in roots but less expressed in other organs. The transcripts of Os*KS3* were detected similarly in roots and stems but poorly expressed in panicles and leaves while Os*KS6* appeared to be highly expressed in roots. In addition, these *KS-like* genes were differentially regulated in imbibed seeds. High accumulation of Os*KS3* and Os*KS4* transcripts were detected in imbibed seeds at 2, 4 and 6 days but no accumulation were found for Os*KS1*, Os*KS2* and Os*KS6* (Pinheiro et al., 2005). These data are controversy to data that *KS* expression shows no tissue specificity in *Arabidopsis thaliana* (Silverstone et al., 1997). In barley a functional analysis of the terpene cyclase encoded in the Hv*CPSL1* and Hv*KSL1* genes sequenced by (Spielmeyer et al., 2004)) remains to be done. It has been demonstrated that ent-*KS1a* and ent-*KSL4*,now confirmed to be from a single gene (Accession number: AK370792), were upregulated by *P. indica* at 3 and 7 days after infection (dai) (Schäfer et al., 2009). 83% similarity was shared between Hv*AK370792* and the rice Os*KSL1* gene (Figure 3-12). The wealth of information about *KS* genes available from rice, Arabidopsis and other plant species will facilitate the elucidation of barley KSL genes. However, further investigations are required to demonstrate the biological role of HvKS-like proteins.

4.5.2 Insight into the evolution of plant kaurene synthase genes

Since 2004, a lot of emphasis was put to investigate terpene cyclases. Copalyl diphosphate synthase and kaurene synthase are the first two enzymes participated in the GA biosynthesis. Terpene cyclases play a key role as a gatekeeper, restricting the location and activity of the early stages of GA biosynthesis (Prisic and Peters, 2007). GA biosynthesis is a complex but important pathway which is linked tightly to its metabolism, while GA metabolism might integrate with other signaling pathways to regulate plant growth and development (Olszewski et al., 2002).The more and more studies has accelerated the understanding of GA signaling components and will be essential in revealing differences in the relative importance of these components among species. Terpene cyclases have been isolated in *Arabidopsis thaliana*, pumpkin (*Cucurbita maxima)*, maize (*Zea mays)*, rice (*Oryza sativa*), cultivated lettuce (*Lactuca sativa*), stevia (*Stevia rebaudiana*), grand fir (*Abies grandis*), wheat (*Tritium aestivum*) and barley (*Hordeum vulgare)* as well as moss (*Physcomitrella patens*) (Otomo et al., 2004; Spielmeyer et al., 2004; Pinheiro et al., 2005; Hayashi et al., 2006; Tomonobu et al., 2009)**.** Phylogenetic analyses revealed that KS-like proteins are divided into monocot and dicot groups (Sakamoto et al., 2004) and indicated that rice and barley share more similarities in their terpene cyclase sequences. In rice, the *KS- like* genes are well studied and at least five *KS-like* genes (Os*KS1*, Os*KS2*, Os*KS3*, Os*KS4*, Os*KS6*) were identified (Pinheiro et al., 2005). Comparisons of terpene cyclase gene sequences between gymnosperms and angiosperms showed a high conservation suggesting limited molecular evolution (Keeling et al., 2010). The formation of

kaurene from GGDP in angiosperms is catalyzed by copalyl diphosphate synthase carrying the DXDD motif and kaurene synthase supplied with the DDXXD motif (Yamaguchi, 2008). In contrast, the formation of kaurene in fungi (*Gibberella fujikuroi*) (Toyomasu et al., 2000), *Phaeosphaeris species* L487 (Kawaide et al., 1997) and in the moss land plant *Physcomitrella patens* (Brophy et al., 2000; Hayashi et al., 2006; Anterola and Shanle, 2008) is catalyzed by bifunctional diterpene synthases. The former enzymes contain two active sites, namely the N-terminal active site containing a conserved DXDD motif (Prisic et al., 2007) and C-terminal harboring a conserved DDXXD motif (Christianson, 2006). My studies suggest, in addition to Hv*AK370792*, the existence of three additional KSL genes (Hv*KSL1*, Hv*AK362172*, Hv*KS2*) (Figure 3-12) with varying degrees of sequence relatedness to Hv*AK370792*. Multiple alignment of the amino acid sequences revealed that these genes contain the conserved DDXXD domain, which is supposed to function as the divalent metal ion-diphosphate complex binding site in terpene cyclases (Pinheiro et al., 2005). Additionally, a small stretch of identical amino acids near the SAYDTAW motif was found in the N-terminal region of barley *KS-like* genes, which is in agreement with the previous demonstration that the conserved motif is required to maintain cyclase activity of plant diterpene cyclase (Kawaide et al., 2000).

4.5.3 Subcellular localization of Kaurene synthases

The subcellular localization of proteins involved in GA biosynthesis helps us to understand their functions in barley. In Arabidopsis, the proteins participating in the three steps of GA biosynthesis have been analyzed (Helliwell et al., 2001). The first two

enzymes of the GA pathway, AtCPS and AtKS, were proven to be located in the plastid stroma without cleaving its leader sequence. This is controversy to the previous report that the N-terminal targeting peptide of AtCPS was cleaved off when it was imported into the chloroplast (Sun and Kamiya, 1994). AtKO1 is targeted to the outer envelope membrane of plastids although the P450 allene oxide synthase in barley has been shown to be localized within chloroplasts (Maucher et al., 2000). P450 allene oxide synthase which metabolize fatty acid hydroperoxides to allene oxides is the first enzyme involved in the jasmonic acid (JA) formation (Maucher et al., 2000). Their data further suggested that some KO is located in the endoplasmic reticulum. However, AtKO1 was confirmed to provide an important link between the plastid and the endoplasmic reticulum-located steps of the GA biosynthesis pathway. The localization of the enzymes involved in the GA biosynthesis pathway in Arabidopsis is concluded in Figure 4-1 (Helliwell et al., 2001). By applying ChloroP, all barley terpene cyclase proteins, except the *syn*-CPS were predicted to have a plastid transit peptide (Figure 4-1). It implies that barley CPS and KS proteins are also localized in chloroplasts. However, further investigations are required to independently confirm the subcellular localization of barley CPS and KS proteins.

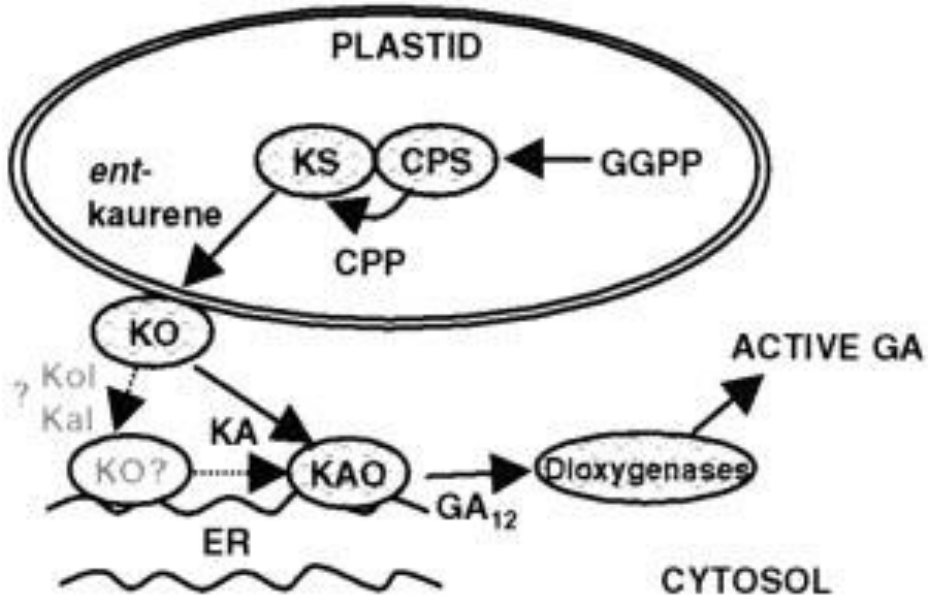


Figure 4-1. Localization of enzymes and distribution of intermediates of the GA biosynthesis pathway (Helliwell et al., 2001).

4.6 *KS-like* gene expression and *P. indica* colonization

4.6.1 *KS-like* genes are induced during *P. indica* colonization

My and previous studies (Schäfer et al., 2009) demonstrated that barley *KS-like* genes were up-regulated in barley roots during colonization by *P. indica*. The transcript accumulation of HvAK370792 was enhanced at 3 and 7 dai (Figure 3-14A), which is in agreement with previous observations (Schäfer et al., 2009). In addition, the Hv*KSL1* transcript accumulation increased at 3 dai, while Hv*KS4* transcription increased at 1 and 3 dai (Figure 3-14B, C). In general, genes expression profiles affected by *P. indica* are associated with extracellular colonization at 1 dai while altered gene transcription at 3 dai or 7 dai are associated with inter/ intra-cellular fungal development. The induction of defence or stress–responsive genes at 1 dai

might indicate the activation of the plant innate immune system by *P. indica* (Schäfe et al., 2009). Whether the kaurene synthase like genes, for instance, Hv*KS4* gene, contribute to plant innate immunity or play a role in the other (phytohormone) pathways remains to be answered. In rice, several CPS-like and KS-like genes were identified and only Os*CPS1* and Os*KS1* were involved in GA biosynthesis (Sakamoto et al., 2004). This raises the question what is the function of the other paralogs. Similarly, biochemical and genetic studies are required to answer, 1) whether *P. indica* affects the biosynthesis of GA or other terpenoids, 2) which metabolites are produced by the KS-like proteins, and are these metabolites involved in GA metabolism or other pathways, and3) does *P. indica* affect the synthesis of secondary metabolites involved in GA biosynthesis in barley roots?

4.6.2 *KS-like* genes are required for *P. indica* colonization and might be involved in GA metabolism

Previous work demonstrated that *P. indica* is capable to increase grain yield through stem elongation, growth activations as well as enhances salt stress tolerance, confers disease resistance in barley (Waller et al., 2005). In addition, *P. indica* was also proven to be able to activate kaurene synthase genes (Hv*KS*) expression in barley. Taken together, I hypothesized that *P. indica* might enhance grain yield through activation of the genes involved in GA biosynthesis. Silencing of Hv*AK370792* in barley roots showed tendency of reduced colonization (although these were not significant reduced *P. indica* colonization) which suggests that Hv*AK370792* functions in signaling "pro-fungal" pathways (e.g. GA biosynthesis) rather than toxic

metabolites (e.g. phytoalexins). A similar colonization phenotype was observed in *KSL1* RNAi roots. Those phenotypes might be explained by an altered defense response, as it was demonstrated for barley M117 and M121 mutants that are impaired in GAs synthesis. Both mutants showed a reduced colonization by *P. indica* (Schäfer et al., 2009). Root generation was reduced from *KSL1*-RNAi calli which suggests a key role of KSL1 in root development. Attempts to produce *KS2*-RNAi and *KS4*-RNAi transgenic roots were only partially successful as the majority of calli containing either RNAi gene construct failed to regenerate roots. Silencing of these genes might result in lethality. In addition, *KSL1*-RNAi and *AK370792*-RNAi lines showed less dark green leaves and retarded development as compared to untransformed control plants regenerated from calli. Moreover, reduced spikelet fertility in progeny plants of the *KSL1* RNAi and *AK370792* RNAi was detected. Previous studies reported that active GAs are required for pollen tube growth (Singh et al., 2002), embryo growth and seed development (Swain et al., 1997). Therefore, it is temptive to speculate that the reduced fertilization is due to disturbed pollen germination caused by the reduction of Hv*AK370792* or Hv*KSL1*. Alternatively or in addition, silencing of either gene might affect seed development. It supports the hypothesis that Hv*AK370792* and Hv*KSL1* play a role in GA metabolism and might be involved in the GA biosynthesis pathway.

4.7 Heterologous expression of terpene cyclase of barley

In a number of studies the function of diterpene cyclases was characterized after heterologous expression of glutathion-S-transferase fusions in *Escherichia coli*

(Kawaide et al., 1997; Oikawa et al., 2001). However, encountering general problem is a lack of expression or extremely low expression levels of active proteins as well as their accumulationa inclusion bodies (Clark, 1998; Simmons et al., 1997). In this study, I decided to express all terpene cyclase proteins as N-terminal fusions to a His6-tag. Unfortunately, the immunoblot results demonstrated that all recombinant proteins but HvAK370792 were not detectable by anti-His6-tag polyclonal antibody after expression in bacterial strain BL21 (DE3)-pLysS. However, expression of syn-CPS was detected in the bacterial strain Rosetta. Rosetta host strains are BL21 lacZY (Tuner & trade;) derivatives designed to enhance the expression of eukaryotic proteins that contain codons rarely used by *E. coli.* These strains supply tRNAs for the codons AUA, AGG, AGA, CUA, CCC, GGA on a compatible chloramphenicol resistant plasmid (http://wolfson.huji.ac.il/expression/bac-strains-prot-exp.html). However, the purification results of HvAK370792 and syn-CPS intimated that the targeted protein was partially degraded either by the *E. coli* cells or during the purification procedure though the protein inhibitor PMSF was added during the purification step. There are at least two possible explanations for the phenomenon: 1) The translation did not initiate efficiently and, thus resulted in poor induction of the targeted protein, 2) The plant-derived proteins copalyl diphosphate synthase and kaurene synthase were recognized by the bacterial cell as foreign proteins and digested. A commonly followed strategy for enhancing protein expression is to switch to other expression vectors (pET SUMO expression system) or expression systems,

like yeast or insect cells. It was reported that protein expression in insect cells was successfully used *in vitro* to express a moss diterpene cyclase (Hayashi et al., 2006).

4.8 Future perspectives

My study is a first step to determine the functionof kaurene synthase like genes in phytohormone and phytoalexin metabolism in barley and their participation in the establishment of the mutualistic barley root-*P. indica* symbiosis. My data revealed that the HvA*K370792*, Hv*KSL1* and Hv*KS4* genes were upregulated by *P. indica* and silencing of Hv*AK370792* and Hv*KSL1* reduced the *P. indica* colonization. Further experiments are required to clearly identify whether Hv*AK370792* gene is involved in GA biosynthesis and/or phytoalexin biosynthesis. My work further represents pioneering efforts to establish recombinant protein expression which is a prerequisite for functional studies of the presumably large family of terpene cyclases *in vitro*. Besides the Hv*AK370792* gene, Hv*KSL1*, Hv*AK362172* Hv*KS2* (AK248484*)* and Hv*KS4* were sharing high similarities with Os*KSL1* based on nucleotide sequence comparison. Future studies might concentrate on the quantification of expression levels of all Hv*KSL* but also Hv*CPS* genes during abiotic and biotic stress as well as defined plant developmental processes. This will further enhance our understanding of their function. For instance, the expression of Os*CPS* and Os*KSL1*, which participate only in GA biosynthesis was not changed in UV irradiated rice seedlings while Os*CPS2*, Os*CPS4*, Os*KS4*, Os*KS7* and Os*KS8* were induced (Sakamoto et al,. 2004). Therefore, respective test systems will reveal if some of those KSL or CPS genes in barley are involved in phytoalexin biosynthesis, UV irradiation or

plant-microbe interactions. Functional studies would further require more information on subcelluler localization of these proteins. In *Arabidopsis*，AtKS1 protein is localized in the chloroplast stroma, whereas AtKO1 is targeted to the outer envelope of the chloroplast, while AtKAO1 and AtKAO2 are targeted to the endoplasmic reticulum (Helliwell et al., 2001). Importantly, STARTS developed in this project will support efforts to perform such functional studies in roots and therefore complements our efforts to understand tissue-specificity of plant proteins and metabolic processes. STARTS especially helps to study the participation of plant proteins in the interaction of roots with pathogenic or mutualistic microbes.

5. Summary

In agricultural cropping systems, roots are frequently subjected to a series of abiotic stress as well as biotic stress caused by microbial pathogens and pests which lead seriously yield reduction for crop food. The significance of the root for plant health is in disagreement with the availability of root systems for functional studies. This is more alarming as our knowledge and technology on protective cultivation methods, resistant germplasms, or chemical control strategies to ward off root disease and root stress in crops is limited. Therefore, Stable Root Transformation System (STARTS) was developed in barley on the basis of the conventional stable transformation method. STARTS accelerates functional studies in roots by the continued culture of calli on root induction medium thereby producing large amounts of roots. STARTS allows functional analysis of proteins in roots in about six weeks. The method was proven to be effective to overexpress (*GFP*, *GFP-BI-1*) and silence (Hv*EXPANSIN B1*, *GFP*) genes. Moreover, STARTS was identified to be suitable for the analysis of protein sub-cellular localization by transforming the scutella with a modified version of GFP (mGFP5-ER). Finally, we studied the impact of *BAX INHIBITOR-1* (*BI-1*) overexpression on root colonization by hemi-biotrophic and biotrophic microbes. Results confirmed that STARTS is applicable to study the effect of barley proteins in root–microbe interactions. Most importantly, the method is suitable to pre-screen the effect of candidate genes on root stress resistance and root development.

STARTS provided a good foundation for the second part of my work referred to the function kaurene synthase-like (KSL) genes during barley root colonization by

Piriformospora indica. Sequence alignment of barley *KSL* genes indicated that all these genes contained the aspartate-rich domain DDFFD which is supposed to function as a divalent metal ion-diphosphate complex binding site in terpene cyclases (Pinheiro et al. 2005). Further experiment showed that barley *KS-like* genes (Hv*AK370792*, Hv*KSL1*, Hv*KS4*) were differently up-regulated at 1, 3 and 7 dai and *KS-like* silencing in roots resulted in reduced colonization by *P. indica*. Silencing of the KSL genes Hv*AK370792*, Hv*KSL1* lead to less dark green leaves and slower plant development. Further, I observed reduced spikelet fertility in progenies of RNAi plants heterozygous for Hv*AK370792 and* Hv*KSL1*. The data suggests that HvAK370792 and HvKSL1 are involved in gibberellin (GA) biosynthesis. I was successful in cloning Hv*CPSL1*, Hv*KSL1*, Hv*syn-CPS* and Hv*AK370792* as well as the heterlogous expression of Hv*syn-CPS* and Hv*AK370792* in *E. coli*. This work therefore build the basis to decipher a presumed function of these proteins in GA and/or phytoalexin metabolism.

6. Zusammenfassung

In landwirtschaftlichen Anbausystemen sind Wurzeln häufig abiotischem Stress sowie Pathogene ausgesetzt. Dies führt zu bedeutenden Ertragsverlusten. Das Wurzelwerk ist für die Pflanzengesundheit von essentzieller Bedeutung. Da die Verfügbarkeit von protektiven Anbaumethoden, resistenten Sorten oder effektive chemische Bekämpfungsstrategien gering ist, ist eine Etablierung neuer Methoden für Wurzelanalysen notwendig. Daher wurde in der vorliegenden Arbeit ein stabiles Wurzel-Transformation System (STARTS) auf der Basis konventioneller Transformationsmethoden entwickelt. Infolge der Unterdrückung der Sprossentwicklung von Kalli und der gleichzeitigen Stimulierung der Wurzelentwicklung erlaubt STARTS die Produktion großer Mengen von Wurzeln. Diese Methode beschleunigt die funktionelle Analyse von Proteinen in Wurzeln innerhalb von 6 Wochen. STARTS wurde erfolgreich in der Überexpression von grün fluoreszierendem Protein (GFP) und der Unterdrückung der Expression des Gersten Expansin B1- (Hv*EXPANSIN B1*) angewendet. Darüber hinaus erwies sich STARTS als geeignet für die Analyse der subzellulären Lokalisation von Proteinen. Zudem erlaubte STARTS die Untersuchung von Pflanzen-Pilz Interaktionen. Hier wurde der Einfluss der Überexpression von Gersten *BAX INHIBITOR-1* (*BI-1*) in Wurzeln auf die Besiedlung durch hemibiotrophe bzw. biotrophe Wurzelmikroben untersucht. Meine Untersuchungen belegen, dass diese Methode für funktionelle Untersuchungen von Geneffekten auf Stressresistenz und Wurzelentwicklung geeignet ist.auf .

STARTS bot eine gute Grundlage für den zweiten Teil dieser Arbeit. Dieser bezog sich auf die Untersuchung der Funktion Kaurensynthase (KS)- ähnlicher Porteine während der Wurzelbesiedlung durch den mutualistischen Pilz *Piriformospora indica*. Sequenzvergleich von Gerste KS- ähnlichen Genen zeigte, dass diese Gene des Aspartat-reiche Domäne, die DDFFD enthalten, welche als Bindungsstellen für zweiwertige Metallion-Diphosphat-Komplexe in Terpencyclasen fungieren (Pinheiro et al., 2005). Darüber hinaus zeigten meine molekularbiologischen Experimente, dass Gerste KS- ähnlichen Gene (Hv*AK370792*, Hv*KSL1*, Hv*KS4*) zu 1, 3 und 7 Tagen nach Inokulation mit *P. indica* in Wurzeln induziert waren. Die Unterdrückung der entsprechenden Genen in STARTS regenerierten T1-Pflanzen durch Expression von HvAK370792-RNAi und HvKSL1-RNAi Konstrukten resultierte in einer geringeren Besiedlung durch *P. indica* , in einem verlangsamten Wachstum und der Entwicklungblassgrüner Blätter . Darüber hinaus wurde eine reduzierte Ährenfruchtbarkeit der heterozygoten Nachkommen für beide RNAi-Linien (HvAK370792-RNAi, HvKSL1-RNAi) beobachtet. Diese Daten unterstützen die Vermutung, dass HvAK370792 und HvKSL1 in der Gibberellinsäure-Biosynthese beteiligt sind.Als Teil dieser Arbeit wurden HvCPSL1, HvKSL1, Hvsyn-CPS und HvAK370792 erfolgreich kloniert sowie die rekombinanten Expression von Hvsyn-CPS und HvAK370792 in *E. coli* durchgeführt. Diese Arbeit bietet somit eine gute Grundlage für zukünftige Studies über die mögliche Beteiligung dieser Proteine im Gibberellinsäure bzw. Phytoalexin-Metabolismus. Allerdings, für Enzym-Assay

sind weitere Expression und Reinigung Schritte der HvCPSL1, HvKSL1 Proteine erforderlich.

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Declaration

I declare that the dissertation here submitted is entirely my own work, written without any illegitimate help by any third party and solely with materials as indicated in the dissertation.

I have indicated in the text where I have used texts from already published sources, either word for word or in substance, and where I have made statements based on oral information given to me.

At all times during the investigations carried out by me and described in the dissertation, I have followed the principles of good scientific practice as defined in the "statutes of the Justus Liebig University Giessen for the Safe guarding of Good Scientific Practice".

Signature:

Date:

Acknowledgements

There is one sentence goes around my ear when I began to write down this paragraph: “There is no royal road to science, and only those who do not dread the fatiguing climb of its steep paths have a chance of gaining its luminous summits”. Absolutely, Karl Marx is right! However, I would like to say without the helping hands given by a number of people from whom I have benefited directly or indirectly I can not move ahead……

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The curriculum vitae was removed from the electronic version of the paper.