Cent. Eur. J. Biol.
DOI: 10.2478/s11535-011-0009-9

VERSITA

Central European Journal of **Biology**

Increased vegetative development and sturdiness of *storekeeper*-transgenic tobacco

Research Article

Moritz Bömer[1,#], Joachim F. Uhrig[2], Guido Jach[3], Kai J. Müller[4,*]

[1]*Westphalian Wilhelms-University Münster, Institute for Biochemistry and Biotechnology of Plants (IBBP), 48143 Münster, Germany*

[2]*University of Cologne, Institute of Botany, 50674 Cologne, Germany*

[3]*Phytowelt GmbH, R&D Facilities, 50829 Cologne, Germany*

[4]*Fraunhofer Institute for Molecular Biology and Applied Ecology (FhG IME), 52074 Aachen, Germany*

[#]*present address: School of Biological Sciences, Royal Holloway University of London, TW20 0EX Egham, Surrey, United Kingdom*

Received 27 July 2010; Accepted 04 January 2011

Abstract: The STOREKEEPER (STK) family of DNA-binding proteins work as transcription factors and the ectopic expression of two *stk*-like genes from *Arabidopsis thaliana*, *stk01* (At1g61730) and *stk03* (At4g00238), in tobacco increased the number of vegetative internodes and promoted plant and leaf size, stem diameter and sturdiness. The development of these plants started with rosette formation while pronounced shoot elongation and flowering was delayed. Moreover, when the STK01 and STK03 proteins were fused to the Herpes Simplex Virus VP16 transcriptional activation domain and expressed in tobacco the vigorous storekeeper-phenotype did not appear indicating that transgenic STK-like proteins in part worked as repressors of tobacco reproductive development. Furthermore, Yeast Two-Hybrid screenings proved that STK01 and STK03 can form homodimers and heterodimers with further members of the STK-like family. Therefore, we assume that interactions between transgenic Arabidopsis STKs and resident tobacco STKs could have contributed to the observed developmental changes in transgenic tobacco. Our findings open up promising applications for over-expression of *stk*-like genes in crops that benefit from increased sturdiness and vegetative organ development, such as tobacco in molecular farming approaches, biomass-based energy crops and medicinal plants that produce bioactive compounds in leaves.

Keywords: *Storekeeper • GEBP-like (GPL) proteins • Sturdiness • Flowering time • Rosette • Biomass*

1. Introduction

Genetic engineering in order to prolong the vegetative development of a plant is an attractive business since several crops profit from increased biomass, e.g. crops used for fibre production and molecular or energy farming [1,2]. One aspect that coincides with increased biomasses of annual crops is the reduced capacity of the plant to withstand environmental challenges such as storms and heavy rains that often cause lodging. Therefore, a favourable trait is an increased sturdiness of the crop, *i.e.* an enlarged or more robust stem in relation to plant height [3]. In the last century, plant breeding efforts to generate sturdier crops that are also able to carry heavier fruit loads were mainly generated by introgression of dwarf mutations into commercial varieties, e.g. wheat [4]. Obviously, dwarfism is a counterproductive trait when increased biomass is one goal of a breeding program and here we describe an option to genetically engineer both prolonged vegetative development and sturdiness of tobacco by single gene manipulations using *storekeeper-(stk-)* like genes from Arabidopsis.

* *E-mail: mueller@molbiotech.rwth-aachen.de*

The plant specific STOREKEEPER protein was first reported to bind to the B-box of promoters of potato *patatin* genes that participate in the regulation of storage protein production in tubers [5]. Subsequently, GEBP (*Glabrous1* enhancer binding protein) of Arabidopsis was found to be a STK-like protein involved in epidermal cell determination. Furthermore, *GEBP* was up-regulated nearly 20-fold in *knox1* homeobox gene over-expressing *35S::KNAT1* plants and shut down in the corresponding *brevipedicellus (bp)* Arabidopsis mutant indicative for a broader role of *GEBP* in the regulation of Arabidopsis development. In addition, 21 sequences were identified making up the STK-like family of DNA binding proteins in Arabidopsis. More important with respect to the data presented, the over-expression of *GEBP* in Arabidopsis did not result in a phenotype deviating from the wild type [6]. Recently, GEBP and three STK-like proteins (At2g25650, At2g36340 and At5g14270, also termed GEBP-like proteins: GPL1, 2 and 3) were shown to form homo- and heterodimers *via* unconventional leucine zipper motifs and to participate in the regulation of cytokinin response in *Arabidopsis thaliana* [7]. We have started characterizing *stk*-like genes after screening Arabidopsis expression libraries in the Yeast One-Hybrid system using as a bait 700 bp of the 5´-silencer region within intron 2 of the Arabidopsis floral homeotic gene *agamous* (*ag*; [8]). This screen resulted in the isolation of four *stk*-like genes (At1g61730 [*stk01*], At4g25210 [*stk02*], At4g00238 [*stk03*] and At4g00270 [*GEBP*]) when the plant-specific GAGA-factor BPC3 (At1g68120) was co-expressed in yeast (J.F. Uhrig and K.J. Müller, unpublished data). Here we demonstrate that the ectopic expression of STK01 and STK03 in tobacco increased stem diameter, foliage, plant height and sturdiness. In contrast, when the two STK-like proteins were fused to the VP16 transcriptional activation domain and expressed in tobacco, the phenotypes remained wild type. Furthermore, protein interaction screenings in yeast indicated that STK proteins can dimerize and we speculate that these characteristics of the STK-like proteins contributed to the phenotypes observed in tobacco upon ectopic expression.

2. Experimental Procedures

2.1 Gene cloning

For *stk01* (At1g61730) and *stk03* (At4g00238) cDNA fragments were amplified by RT-PCR from Arabidopsis total seedling RNA (4 weeks after germination) using 5´- and 3´- primers elongated by *Nco*I and *Xba*I restriction sites, respectively, (sk01_fw: AAA ACC ATG GGA ATG ACG AAG AAA CTC AAT CCA CTG; sk03_fw: AAA ACC ATG GCT TCT TTG GAA AAT CCA GCA) (sk01_bw: AAA ATC TAG ACT ATG TAT CTA ATG GCT TGT TCT TA; sk03_bw: AAA ATC TAG ATT AGT TGG TTT GAG TAA GCA CTG AAG) and cloned in 5´ to 3´ orientation using *Nco*I and *Xba*I under control of the CaMV 35S promoter and terminator sequences in pRT104 [9]. Sequences were authenticated according to database accessions for At1g61730 and At4g00238. To attach to the 5´-end of the *stk01* and *stk03* cDNAs the sequence encoding the HSV V16 transactivation domain it was amplified by PCR from the plasmid pcDNA3-VP16 (kindly provided by Dr. Andreas Hecht, Institute of Molecular Medicine and Cell Research, University of Freiburg, Germany) using the *Nco*I-elongated primers VP16-5 (AAA ACC ATG GGT GAC GAG CTC CAC TTA GAC GGC) and VP16-3 (AAA ACC ATG GCA CCA CCG TACT CGT CAA TTC CC) and cloned by *Nco*I. For the derived clones the orientations of VP16 sequences were checked by PCR and sequencing, thus confirming the exact fusions of the VP16-STK01 and VP16-STK03 peptides. Subsequently, *35S::stk01::T*, *35S::stk03::T*, *35S::vp16-stk01::T* and *35S::vp16- stk03::T* fragments were released from the pRT104 vectors by *Pst*I and cloned into the unique *Pst*I site of the multiple cloning site of the binary vector pCambia2300 (http://www.cambia.org/daisy/cambia/585#dsy585_Description).

2.2 Stable and transient plant transformation and protoplast assays

The binary pCambia2300 constructs were electroporated in *Agrobacterium tumefaciens* EHA105 and prepared for standard leaf disc transformation of *Nicotiana tabacum* Petite Havana SR1 [10] and the Arabidopsis floral dip transformation [11]. Tobacco *Nicotiana tabacum* Petite Havana SR1 protoplasts were prepared and transiently transformed [12] and GUS assays were performed according to established protocols [13].

2.3 Standard molecular biology

The Southern and northern analyses of selected transgenic plants were carried out according to [14]. RT-PCR expression analysis was realized with Superscript II (Invitrogen, Karlsruhe, Germany) according to the manufacturers' instruction, oligo dT primer described [15] and 30 cycles using the gene specific primers that amplified DNA fragments of expected sizes for the S-plants and the VP16-5 primer together with the 3´ gene specific primer for the VS-plants. Primers of the tobacco *gapdh* gene to normalize the RT-PCRs were GAPDHfw (AGC TCA AGG TTA AGG ATG AC) and GAPDHbw (TGG CCA AGG GAG CAA GGC).

2.4 Cloning of Gus reporter plasmid

The *CaMV*35S::Gus::Terminator cassette was released by *Pst*I from pRT103GUS [16] and inserted into the *Pst*I site of pUC19 to obtain pUC103GUS. Subsequently, the 35S promotor was removed from pUC103GUS by *Hind*III and *Xho*I and the vector re-ligated to form pUC103GUSD35S after a Klenow fill-in reaction. In a next step the vector pUC103GUSD35SD3´PCS was constructed by removing *Pst*I to *Sac*I restriction sites by restriction and Klenow enzyme exonuclease reaction prior to re-ligation. Finally, the 35S promoter was re-inserted by *Hind*II-*Nco*I to obtain pUC103GUSDPCS (35SGus in Figure 3). Based on this vector the 35SminGUS construct was obtained by removing the 35S enhancer through restriction by *Eco*RV and *Hinc*II followed by re-ligation.

2.5 Yeast Two-Hybrid Screening

Yeast strains AH109 [17] and Y187 [18] were maintained in standard yeast full media or selective drop-out media (Clontech) using standard conditions. Transformation of plasmids into yeast was done according to the LiAc transformation method [19]. STK01 and STK03 constructs were cloned into the bait vector pAS2.1 (Clontech), and yeast-2-hybrid screening of an Arabidopsis cell suspension culture cDNA library in pACT2.1 [20] was carried out according to [21].

3. Results

3.1 Analyses of primary tobacco *storekeeper* transformants

From tissue culture we transferred a total of 11 S1 (*35S::stk01*), 21 S3 (*35S::stk03*), 25 VS1 (*35S::vp16stk01*) and 14 VS3 (*35S::vp16stk03*, Figure 1a) independent kanamycin-resistant tobacco transformants (T_0) to the greenhouse. The most obvious trait of the primary *storekeeper*-transgenic S-plants was a substantially increased stem diameter in comparison to wild type tobacco (Figure 1b). Furthermore, the T_0-generation of S1- and S3-plants developed increased numbers of foliar leaves. In order to confirm transformation events and expression levels of the transgenes at least four plants for each construct were subjected to Southern and northern hybridizations (Supplemental Figure 1): The selected plants derived from independent transformation events each carried one to three T-DNA insertions. However, levels of transgene expression were detectable in only few cases by northern experiments, namely S1-4, S1-11, S3-1, S3-9, S3-10, VS1-5, VS1-2, VS1-9, VS3-5. As exemplified for the randomly selected S1- and S3-plants transgene expression levels were not correlated with increased stem diameters (Table 1).

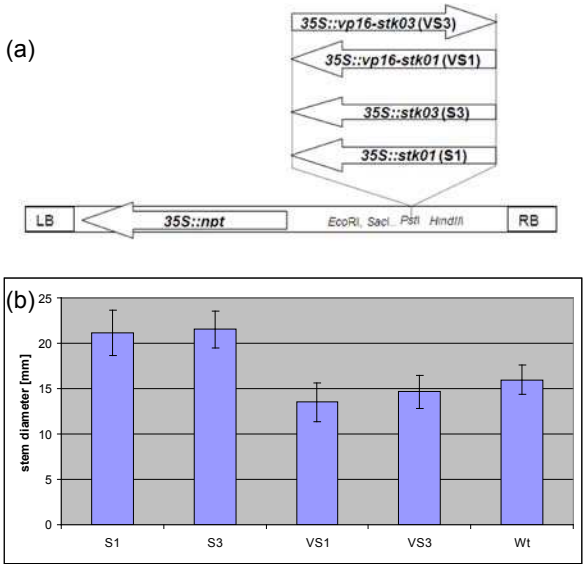


Figure 1. DNA-constructs and stem diameters of primary storekeeper-transgenic tobacco plants. (a) Schematic display of the T-DNAs transformed in tobacco based on the binary vector pCambia 2300. LB and RB: left and right T-DNA borders, respectively; npt: neomycinphosphotransferase. 35S: *CaMV* 35S promoter. (b) Means of stem diameters of primary tobacco transformants (T_0-plants). 12 wild type plants were measured in comparison to 11 S1, 21 S3, 25 VS1 and 14 VS3 plants. Individual plant stem diameters were determined 1 cm above ground as means of 3 measurements with a digital gauge.

Plant lines	Stem diameter (mm)	Transgene expression detection (northern)
S3- 1	18.8	+ +
S3- 5	20.6	-
S3- 9	23.9	+
S3- 10	20.7	+ +
S1- 4	20.1	+
S1- 5	23.1	-
S1-11	20.5	+
WTmax	18.0	n.d.
WTmin	13.4	n.d.

Table 1. Selected T_0 S-plant lines, stem diameters and expression levels of transgenes. The levels of *storekeeper* transgene expression (see Supplemental Figure 1) were not correlated with increased stem diameters of *35S::stk01* (S1) and *35S::stk03* (S3) plants.

3.2 Developmental features of *35S::stk01* and *35S::stk03* plants

The growth and development of the T_1- and T_2-progeny of *storekeeper*-transgenic tobacco plants was monitored and compared: VS-plants developed similar to the wild type but shoot elongation was blocked in S1- and S3-plants until VS- and the wild type plants flowered (Figure 2a). Subsequently, shoot elongation also became obvious for S-plants: S1- and S3-progenies grew taller, flowered 3-4 weeks later than VS-plants and wild type, carried more and enlarged foliar leaves and initially no prominent lateral shoots (Figure 2b). A gross inspection of shoot cross sections showed that all tissues of the stems in the S-plants were enlarged in comparison to wild type tobacco (Figure 2c). Further T_1- and T_2-progenies were cultivated to assure the data obtained yet (Table 2): S-Plants developed 22-25 leaves before flowering while VS-plants and the wild type carried 14-15 foliar leaves. Moreover, when lateral shoots emerged on S-plants later in development they carried 10-13 leaves while in wild type plants there were only 5-7 (data not shown). Furthermore, it was confirmed that the expression level of the transgenes in S-plants was not correlated with the severity of the phenotypes. The ratio shoot height over stem diameter revealed that most S-Plants developed an increased sturdiness (ratios ranging from 49.2 to 61.2) in comparison to VS-plant and wild type tobacco (ratios ranging from 59.4 to 80.4; Table 2). Thus, the S-plants not only increased foliage but also sturdiness in comparison to wild type and VS-plants.

In contrast to S-plants the VS-plants did not develop phenotypes deviating from wild type tobacco and thus we wanted to know whether the VP16-STK fusion was functional. Transient transformation experiments using tobacco protoplasts revealed that the fusion protein of the HSV-VP16 transcriptional activation domain to STK01 was able to strongly activate transcription of the Gus-reporter gene when a *Cauliflower Mosaic Virus* 35S minimal promoter was cloned in *cis* (Figure 3). We therefore concluded that the VP16-STK fusions were also functional peptides in the transgenic VS-tobacco plants.

Furthermore, we analysed the expression of putative target genes in our *storekeeper*-transgenic tobacco plant material by RT-PCR but neither identified *mis*-regulated expression of *Nicotiana agamous* (*NAG* [22]) nor saw alterations of expression levels of *patatin*- and *centroradialis*-like genes (data not shown; see introduction and discussion).

3.3 STOREKEEPER-like proteins interact

In order to identify potential interaction partners of the STK proteins we performed Yeast Two-Hybrid-screenings using as baits STK01 and STK03: Both proteins were able to form homodimers in this system and, in addition, interacted

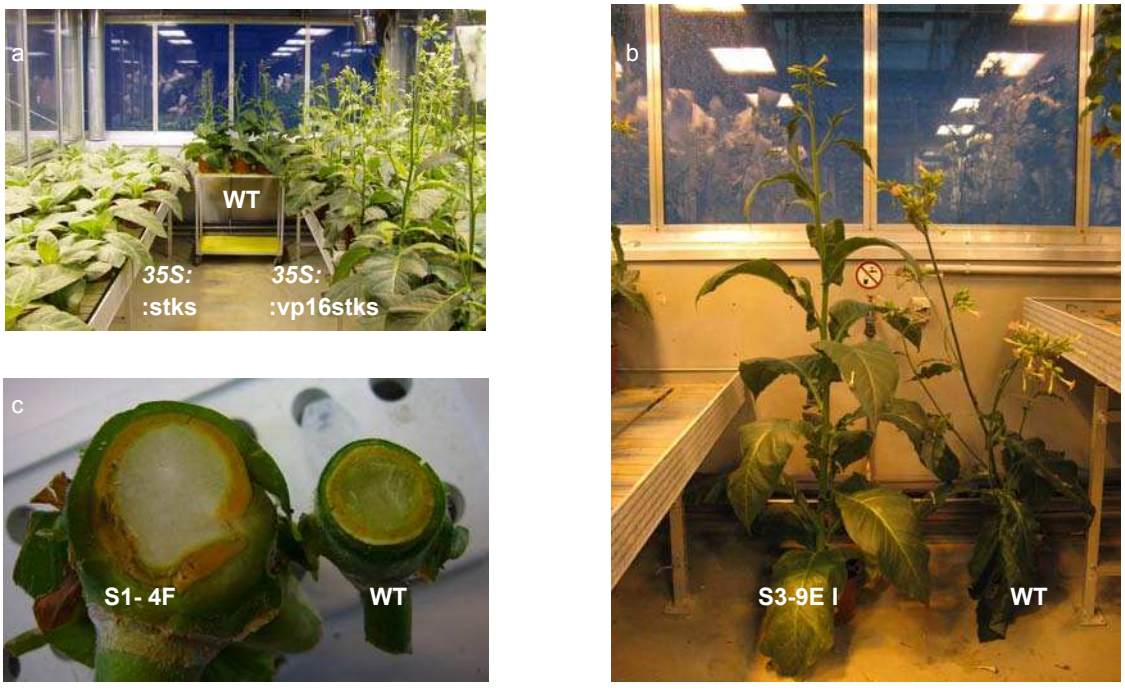


Figure 2. Phenotypes of *storekeeper*-transgenic tobacco. T_1-progenies of T_0-plants positive in northern and Southern experiments were grown in comparison to wild type plants. In (a) an overview is given 80 days post germination with S-plants to the left, VS-plants to the right and wild type in the middle. In (b) and (c) S-plants are depicted in comparison to the wild type 120 days after germination. The cross section in c was taken 1 cm above ground.

Plant line	Time to flowering (days after germination)	Leaves to inflorescence	Shoot height (mm)	Stem diameter (mm)	Shoot height / Stem diameter	Transgene expression
S1-4F I		24	1470	26.1	56.3	
S1-4F II	~100	22	1450	23.7	61.2	-
S1-4F III		23	1500	27.9	53.8	-
S1-4F IV		25	1320	22.7	58.2	
S1-11B I		25	1470	27.2	54	
S1-11B II	~100	24	1280	23	55.7	
S3-1 F I		23	1530	26.3	58.2	
S3-1 F II	~100	23	1520	23.5	64.7	
S3-9 E I		23	1670	31.8	52.5	
S3-9 E II	~100	25	1460	29.7	49.2	
VS1-2 A		15	1250	17.2	72.7	
VS1-2 B	~ 70	14	1120	17.5	64	
VS1-2 C		15	1240	18.1	68.5	
VS1-2 D		14	1140	19.2	59.4	-
VS3-5 A		15	1000	15.3	65.4	-
VS3-5 B	~ 75	15	1030	15	68.7	-
VS3-5 C		15	960	15.2	63.2	
VS3-5 D		14	1230	15.3	80.4	-
WT-A		15	1040	15.4	67.5	-
WT-B	~ 75	14	1100	14.9	73.8	-
WT-C		15	1150	18	63.9	-

Table 2. Phenotypic traits of S- (T_2) and VS- (T_1)- tobacco plants. The phenotypic traits were measured during winter 2007/2008 in the greenhouse: Flowering time "deadline" was the appearance of the first tobacco flower per plant and shoot height was measured when the inflorescence was fully developed. The stem diameter was calculated as the means of three measurements with a digital gauge 1 cm above ground. Transgene expression was determined by RT-PCR (30 cycles). A standardization for S- and VS-plants was done prior by detection of a uniform expression level of the tobacco *gapdh* gene in the relevant samples (Supplemental Figure 2).

with STK05. Furthermore, STK03 interacted with STK06 and further proteins from an Arabidopsis expression library were identified as potential interaction partners of STK01 and STK03 that could serve as future candidates to study their effects on vegetative plant development upon over-expression or knock-down (Table 3).

4. Discussion

We demonstrated that in tobacco the ectopic expression of two *storekeeper*-like genes from Arabidopsis initially induced the development of a rosette followed by pronounced internode growth and development of vegetative organs resulting in plants taller than the wild type and with increased foliage and sturdiness. In contrast, over-expression of the STK-like proteins fused to the VP16 transcriptional activation domain did not affect tobacco development.

From the phenotypes of *storekeeper*-transgenic plant lines S1 and S3 we concluded that STK-like proteins participated in the repression of flowering time and / or in the activation of vegetative growth that lead to increased stem diameters and more internodes and leaves. In

Bait	Isolated Gene	N° of clones	Description
STK01	**At1g61730**	**15**	**STK01**
	At1g44810	**2**	**Storekeeper-like („STK05“)**
	At4g00390	**1**	**Storekeeper-like („STK06“)**
	At4g30200	2	similar to VIN3 (VERNALIZATION INSENSITIVE 3)
	At2g20310	2	RIN13 (RPM1 INTERACTING PROTEIN 13)
	At2g03400	1	hypothetical protein
	At2g03390	1	uvrB/uvrC motif-containing protein
	At2g35920	1	ATP-dependent RNA helicase A like protein
STK03	**At4g00238**	**9**	**STK03**
	At1g44810	**2**	**Storekeeper-like („STK05“)**
	At5g22000	1	RING-H2 finger protein RHF2a
	At1g49475	1	similar to Transcriptional factor B3

Table 3. Interactions of STK01 and STK03 revealed by Yeast Two-Hybrid screens. Using STK01 and STK03 as baits in the Yeast Two-Hybrid system interacting proteins were identified and their identities are displayed. From the numbers of clones isolated for each gene we concluded that STK01 and STK03 preferentially formed homodimers. However, heterodimerization with other STK-like proteins was observed and, in addition, we present potential interaction partners of STK01 and STK03 other than STK-like proteins that will serve as targets to study their impact on vegetative plant development.

this sense, the VS-plants suggested that repression of flowering time in S-plants was not mediated through the activation of a floral repressor. A further intriguing finding was the rosette-like phenotype in S-plants at early stages of development that prompted us to the speculation that *stk*-like genes could be involved in the establishment of the Arabidopsis rosette. However, it became clear that the severity of the phenotypes of the S-plants did not follow the dosage of transgene expression and that expression of the *stk*-transgenes at levels that were undetectable by northern or RT-PCR triggered the developmental consequences mentioned. Therefore, we deduced that a low threshold value existed for the transgenic gene product to realize the heterochronic and morphological responses observed in *35S::stk01- and 35S::stk03*-transgenic tobacco. We and others [7] observed that STK-like proteins were able form homo- and heterodimers and therefore it appeared likely that also interactions occurred between transgenic Arabidopsis and resident tobacco STK-like proteins. To date, ten STK-like sequences have been identified in tobacco and deposited within the TOBFAC database. Thus, a disrupted homeostasis of resident STK-like proteins could have contributed to the generation of the phenotypes of S1- and S3-plants. In the future we will expand target gene screenings of our STK proteins to identify further factors involved in the development of the sturdy S-plants. Putative targets may not only include genes identified by our Yeast Two-Hybrid screenings but also along the pathway of cytokinin signalling since a triple mutant in *stk*-like genes of Arabidopsis was affected in a subset of cytokinin responses [7].

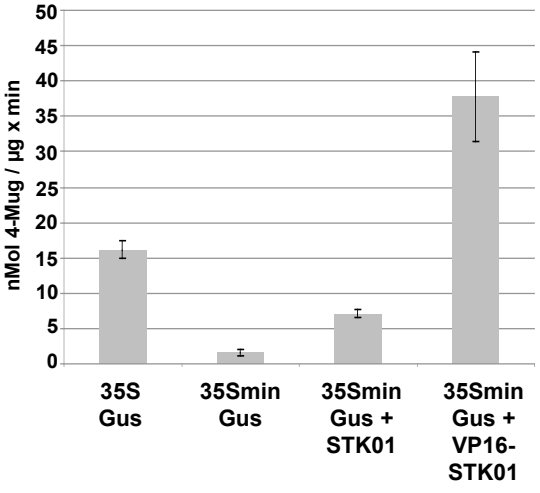


Figure 3. Functional fusion of the HSV VP16 domain to the STK01 protein. Displayed are the results of GUS assays of tobacco protoplasts (means of three measurements) transformed with a plasmid harbouring the full length *CaMV* 35S promoter (35S) and a *CaMV* 35S minimal promoter (35Smin) fused to the *uida* reporter gene (Gus) of *E. coli*. Co-transformations of the plasmid 35SminGus with plasmids driving *stk01* and *vp16::stk01* from the 35S promoter were analysed: The assays revealed that STK01 can slightly activate transcription of the *uida* reporter gene and that the activation was substantially higher for the fusion construct indicative for a functional truncation of STK01 by the HSV VP16 transcriptional activation domain.

One pathway involved in the phase change from vegetative to reproductive development in tobacco is governed by genes homologous to *centroradialis (CEN)*

of *Antirrhinum*: Over-expression of *CEN* in tobacco dramatically prolonged vegetative development and increased foliage [23]. We analysed the expression of four *CEN*-like genes from tobacco (*CET*) in our *storekeeper*-transgenic material but could not detect altered *CET* expression levels in comparison to the wild type. We therefore speculate that the influence of transgenic STK-like proteins on tobacco development was mediated through another pathway involved in flowering time determination or maintenance of vegetative development. Moreover, *mis*-regulation of four *patatin (pat)*-like genes of tobacco [24,25] did not become obvious in our S- and VS-plants. STOREKEEPER is known to bind to promoters of *pat*-like genes [5] and over-expression of *pat*-like genes lead to increased sturdiness in Arabidopsis [3].

We found that the ectopic expression of *stk01* and *stk03* did not result in aberrant phenotypes in transgenic Arabidopsis (data not shown). Similarly, Curaba *et al.* [6] were not able to detect abnormal phenotypes in Arabidopsis upon over-expression of the *stk*-like gene *GEBP*. Apparently, tobacco and Arabidopsis differ in their responses towards over-expressed *storekeeper* gene products. In contrast to the long day plant Arabidopsis tobacco is a day neutral plant and this difference might be a road to be followed in the future experimentally in order to explain the action of over-expressed *stk*-like genes in tobacco. Taken together, our results indicated that especially the increased foliage and sturdiness that we were able to engineer by the use of *stk*-like genes in tobacco can serve as a tool to optimize yield and sturdiness of tobacco in molecular farming approaches. Moreover, genetic engineering with *stk*-like genes can be attempted from now on in crops that would profit substantially from increased foliage and sturdiness, e.g. energy crops and medicinal plants that produce bioactive compounds in leaves.

Acknowledgements

The authors thank Daniela Ahlert for superb technical assistance.

References

[1] Twyman R.M., Stöger E., Schillberg S., Christou P., Fischer R., Molecular farming in plants: host systems and expression technology, Trends Biotechnol., 2003, 21, 570-578

[2] Sticklen M., Plant genetic engineering to improve biomass characteristics for biofuels, Curr. Opin. Biotechnol., 2006, 17, 315-319

[3] Huang S., Cerny R.E., Baht D.S., Brown S.M., Cloning of an Arabidopsis patatin-like gene, STURDY, by activation T-DNA tagging, Plant Physiol., 2001, 125, 573-584

[4] Salamini F., Hormones and the Green Revolution, Science, 2003, 302, 71-72

[5] Zourelidou M., de Torres-Zabala M., Smith C., Bevan M.W., Storekeeper defines a new class of plant-specifc DNA-binding proteins and is a putative regulator of patatin expression, Plant J., 2002, 30, 489-497

[6] Curaba J., Herzog M., Vachon, G., GeBP, the first member of a new gene family in Arabidopsis, encodes a nuclear protein with DNA binding activity and is regulated by KNAT1, Plant J., 2003, 33, 305-317

[7] Chevalier F., Perazza D., Laporte F., Le Hénaff G., Hornitschek P., Bonneville J.-M., et al., GeBP and GeBP-like proteins are noncanonical leucine-zipper transcription factors that regulate cytokinin response in Arabidopsis, Plant Physiol., 2008, 146, 1142-1154

[8] Busch M.A., Bomblies K., Weigel D., Activation of a floral homeotic gene in Arabidopsis, Science, 1999, 285, 585-587

[9] Töpfer R., Matzeit V., Gronenborn B., Schell J., Steinbiß H.-H., A set of plant expression vectors for transcriptional and translational fusions, Nucl. Acids Res., 1987, 15, 5890

[10] Horsch R.B., Fry J.E., Hoffmann N.L., Eichholtz D., Rogers S.G., Fraley, R.T., A simple and general method for transferring genes into plants, Science, 1985, 227, 1229-1231

[11] Clough S.J., Bent A.F., Floral dip: a simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana, Plant J., 1998, 16, 735-743

[12] Locatelli F., Vannini C., Magnani E., Coraggio I., Bracale M., Efficiency of transient transformation in tobacco protoplasts is independent of plasmid amount, Plant Cell Rep., 2003, 21, 865-871

[13] Jefferson R.A., Kavanagh T.A., Bevan M.W., GUS fusions: b-glucuronidase as a sensitive and versatile gene fusion marker in higher plants, EMBO J., 1987, 6, 3901-3907

[14] Sambrook J., Fritsch E.F., Maniatis, T., Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, 1989

[15] Scholz M., Lipinski M., Leupold M., Luftmann H., Ofir R., Fischer R., et al., Methyl jasmonate

induced accumulation of kalopanaxsaponin I in Nigella sativa, Phytochemistry, 2009, 70, 517-522

[16] Töpfer R., Pröls M., Schell J., Steinbiß H.-H., Transient gene expression in tobacco protoplasts: II. Comparison of the reporter gene systems for CAT, NPT and GUS, Plant Cell Rep., 1988, 7, 225-228

[17] Halladay J.P., Craig E.A., Genomic libraries and a host strain designed for highly efficient two-hybrid selection in yeast, Genetics, 1996, 144, 1425-1436

[18] Harper J.W., Adami G.R., Wie N., Keyomarsi K., Elledge, S.J., The p21 Cdk-Interacting protein cip1 is a potent inhibitor of G1 Cyclin-dependent Kinases, Cell, 1993, 75, 805-816

[19] Gietz R.D., Schiestl R.H., Willems A.R., Woods R.A., Studies on the transformation of intact yeast cells by the LiAc/SS-DNA/PEG procedure, Yeast, 1995, 11, 355-360

[20] Nemeth K., Salchert K., Putnoky P., Bhalerao R., Koncz-Kalman Z., Stankovic-Stangeland B., et al., Pleiotropic control of glucose and hormone responses by PRL1, a nuclear WD protein, in Arabidopsis, Genes Dev., 1998, 12, 3059-3073

[21] Soellick T.R., Uhrig J.F., Development of an optimized interaction-mating protocol for large-scale yeast two-hybrid analyses, Genome Biol., 2001, 2, http://genomebiology.com/2001/2/12/research/0052

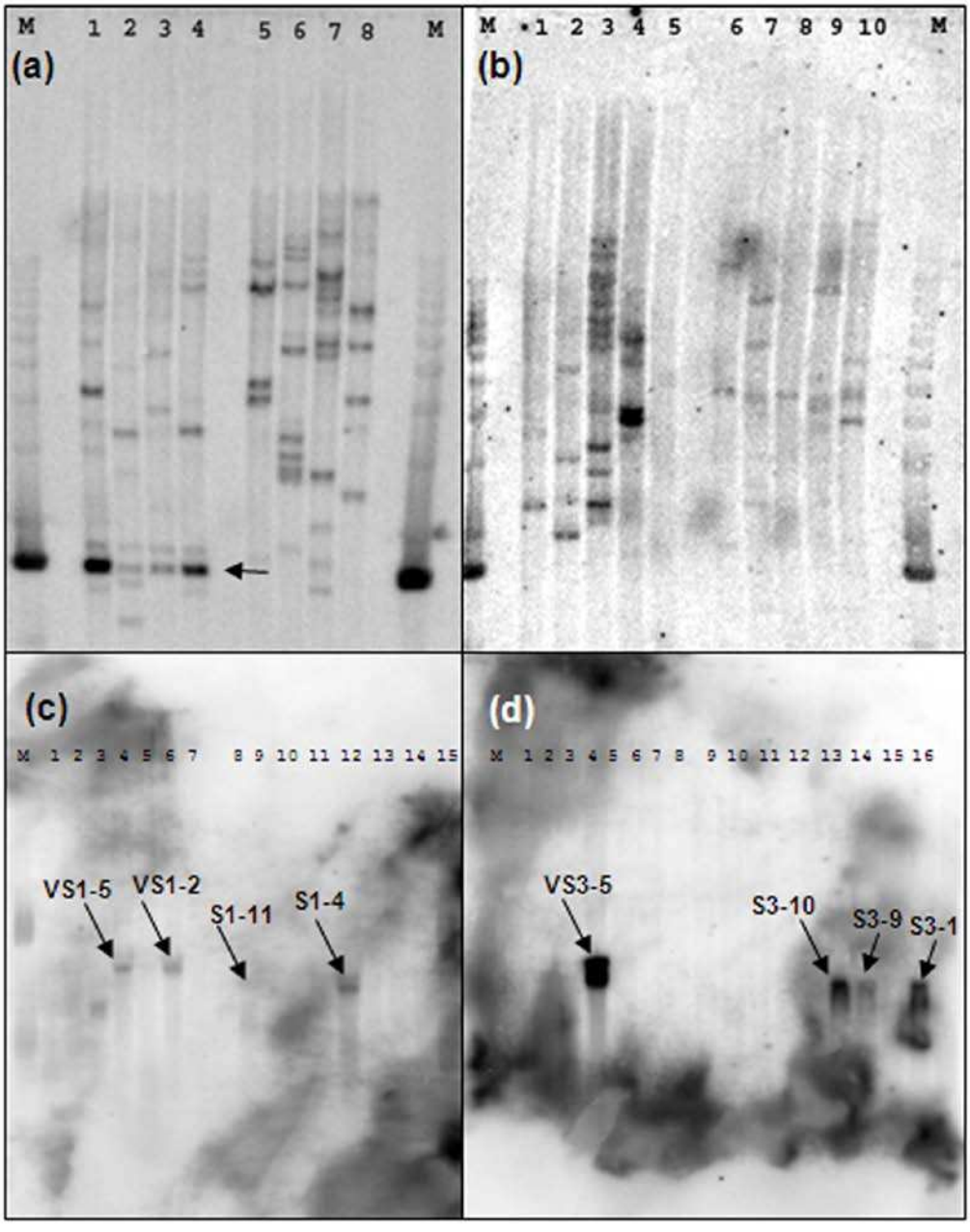
[22] Kempin S.A., Mandel A., Yanofsky M.F., Conversion of perianth into reproductive organs by ectopic expression of tobacco floral homeotic gene NAG1, Plant Physiol., 1993, 103, 1041-1046

[23] Amaya I., Ratcliffe O.J., Bradley D.J., Expression of CENTRORADIALIS (CEN) and CEN-like genes in tobacco reveals a conserved mechanism controlling phase change in diverse species, Plant Cell, 1999, 11, 1405-1417

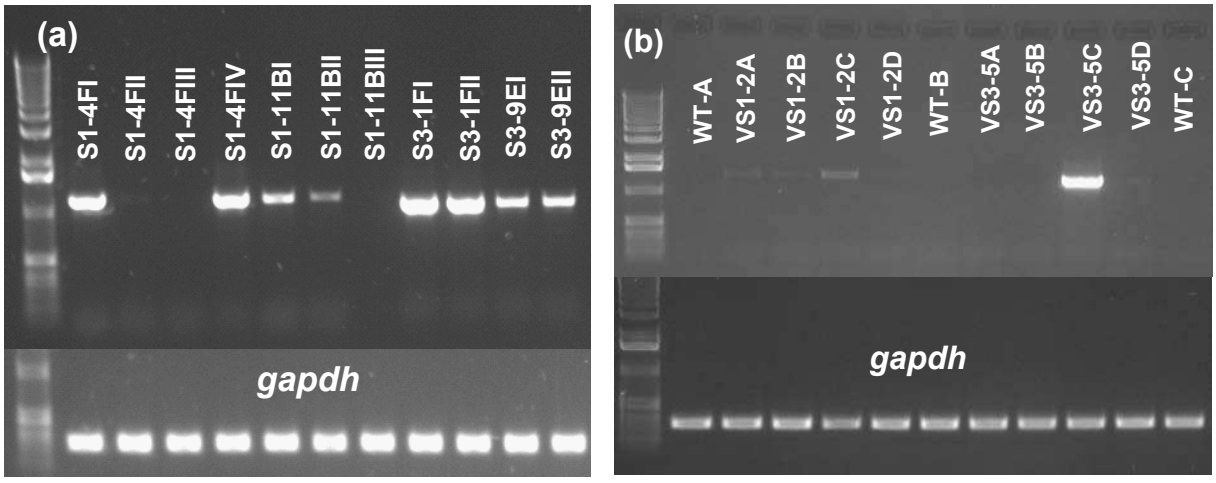
[24] Drews G.N., Beals T.P., Bui A.Q., Goldberg R.B., Regional and cell-specific gene expression patterns during petal development, Plant Cell, 1992, 4, 1383-1404

[25] Dhondt S., Geoffrey P., Stelmach B.A., Legrand M., Heitz T., Soluble phospholipase A2 activity is induced before oxylipin accumulation in tobacco mosaic virus-infected tobacco leaves and is contributed by patatin-like enzymes, Plant J., 2000, 23, 431-440

Appendix



Supplemental Figure 1. Southern- and northern-hybridizations of selected T_0 *storekeeper*-transgenic tobacco. (a) S1/VS1-Southern blot; plant lines with respect to the indicated lanes were: 1: VS1-11; 2: VS1-10; 3: VS1-5; 4: VS1-2; 5: S1-11; 6: S1-4; 7: S1-3; 8: S1-2. 15-20 μg of genomic DNA was digested with *Sac*I; probe corresponded to a 35S-vp16-stk01-T-fragment; M: 1kb ladder (Invitrogen). The uniform band at 1,6 kb (arrow) was due to an internal *Sac*I fragment of the transformed T-DNA construct. (b) S3/VS3-Southern blot; plant lines with respect to the indicated lanes were: 1: S3-1; 2: S3-9; 3: S3-10; 4: S3-12; 5: S3-16; 6: VS3-2; 7: VS3-4; 8: VS3-5; 9: VS3-6; 10: VS3-8. 15-20μg of genomic DNA was digested with EcoRI; probe corresponded to a 35S-vp16-stk03-T-fragment. (c) S1/VS1-northern blot; positive clones are indicated, probe as in a; analysed were 20μg total RNA of the following plants: 1: VS1-1, 2: VS1-10, 3: VS1-9, 4: **VS1-5**, 5: VS1-4, 6: **VS1-2**, 7: VS1-1, 8: **S1-11**, 9: S1-9,10: S1-8, 11: S1-5, 12: **S1-4**, 13: S1-3, 14: S1-2, 15: S1-1. (d) S3/VS3-northern blot; positive clones are labelled, probe as in b; analysed were 20μg total RNA of the following plants: 1: VS3-9, 2: VS3-8, 3: VS3-6, 4: **VS3-5**, 5: VS3-4, 6: VS3-3, 7: VS3-2, 8: VS3-1, 9: S3-18, 10: S3-17, 11: S3-15, 12: S3-12, 13: **S3-10**, 14: **S3-9**, 15: S3-5, 16: **S3-1**.



Supplemental Figure 2. RT-PCR transgene expression analyses of T_1 and T_2 VS- and S-plants, respectively. RT-PCR transgene expression analyses of S- (a) and VS-plants (b) according to Table 2; Plant S1-11BIII died in the course of the experiment and was not included in Table 2. WT-A and WT-B were analysed with VS1 specific primers; WT-C with VS3 specific primers. DNA size marker (left on each gel) was Invitrogen 1kb ladder.