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GABI-FUTURE-Verbundvorhaben:' Funktionelle Genomforschung an Blühgenen zur gezielten genetischen Modifikation des Blühzeitpunkts in Zuckerrübe - GABI - GENOFLOR' (Teilprojekt A)

Förderkennzeichen: 0315058A

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I. Kurze Darstellung zu

1. Aufgabenstellung

Floral transition is a major developmental switch that is tightly controlled by a network of proteins that perceive and integrate developmental and environmental signals to promote or inhibit the transition to flowering. In sugar beet and other crop species, induction and timing of flowering greatly affect yield and the respective crops' potential for cultivation under extensive conditions. In particular, cultivation of sugar beet over winter is expected to result in significant gains in root yield but is not possible in Germany and other central European countries due to induction of flowering by cold temperatures. However, suitable genotypes that on the one hand are resistant to cold induction, but on the other hand can be induced to flower for seed production and multiplication under defined conditions are difficult or impossible to identify by traditional selection and breeding approaches. Therefore, the main objectives of the proposed project are: a) genome-wide identification and expression profiling of floral transition genes in sugar beet, b) RNAi, overexpression and TILLing of select floral transition genes, and c) phenotyping for altered flowering time and bolting resistance to identify viable strategies for the development of winter cultivars. The first-time use of the SuperSAGE technology to study vernalization response in plants was expected to identify novel target genes for genetic modification of floral transition. The participation of two breeding companies allows immediate transfer of know-how, constructs and demonstration plant material for the future development of prototypes for integration into commercial breeding programs.

2. Voraussetzungen, unter denen das Vorhaben durchgeführt wurde

The project was initiated in the light of the competitive nature of the sugar market (sugar beet *vs.* sugar cane), recent and pending changes of its regulation in Europe, and a strong interest by breeding companies to improve the yield potential of sugar beet and its value for alternative uses such as bioenergy production by the development of winter beets. All major sugar beet breeding companies in Europe expressed interest in the project proposal and engaged in discussions with CAU Kiel. Strube and SESVanderHave joined the project. The project fell within the funding module 'GABI-Bridge' and aimed at the transfer, and expansion, of the extensive knowledge on flowering time control in the model plant *Arabidopsis thaliana* to an agronomically important crop species.

3. Planung und Ablauf des Vorhabens

Project A started August 1, 2008 with the hiring of a technician at CAU Kiel. A Ph.D. student was hired on October 1, 2008. Plant material for SuperSAGE had been grown and harvested at CAU Kiel in preparation of the project and was sent to GenXPro ahead of the project start. Sugar beet transformants were produced for all eleven transgene cassettes generated in the project. All transformants were fully molecularly characterized as proposed, which included the expression analysis of all target genes at CAU Kiel. Phenotypically, the transgenic beets derived from seven of the eleven transformation series were fully characterized as proposed, while the phenotypic characterization of the remaining four transformation series is still ongoing. However, it was explicitely stated in the project proposal that the lengthy transformation procedure and the nature of the trait of interest does not allow the phenotypic characterization of all transformants within the project period. The industrial partners therefore had explicitely stated their commitment to continue the phenotypic evaluation of transformants beyond the end of the project period. Rotating annual meetings were held at all project partners.

4. wissenschaftlichem und technischem Stand, an den angeknüpft wurde, insbesondere
• Angabe bekannter Konstruktionen, Verfahren und Schutzrechte, die für die Durchführung des Vorhabens benutzt wurden,
• Angabe der verwendeten Fachliteratur sowie der benutzten Informations- und Dokumentationsdienste

Many of the key genes that control flowering time have been identified and functionally characterized in *A. thaliana* (reviewed in He and Amasino, 2005; Bäurle and Dean, 2006), and plant genome and EST sequencing projects have begun to unveil the presence and evolutionary conservation of these genes across taxa (Albert *et al*., 2005; Hecht *et al*., 2005). For several genes equivalent or related functions in species as diverse as *A. thaliana* and *Oryza sativa* have been demonstrated, but inter-species comparisons also revealed flowering time genes that may have undergone functional divergence during the evolution of flowering behaviour (Putterill *et al*., 2004; Lee *et al*., 2005).

In *B. vulgaris*, the genes and pathways that regulate flowering time were largely unknown at the start of the project. The tendency for early bolting (without vernalization requirement) is under the control of a single dominant gene which was cloned in the PI's group shortly before the start of the project and identified as the pseudo-response regulator gene *BvBTC1*. In consideration of several observations detailed in II.1.1 and our results from the SuperSAGE analysis (s. II.1.1), this gene was included in the functional analysis of floral transition genes in the current project. Furthermore, in biennial beets, induction and timing of flowering depends on vernalization and requires appropriate photoperiodic and developmental conditions, but at the start of the project there was only one published report on a candidate regulatory gene in beet, which was the *A. thaliana Flowering Locus C* (*FLC*) homolog *BvFL1* (Reeves *et al*., 2007). A patent search on the search portals of the European Patent Office and the World Intellectual Patent Organization revealed that *FLC* and a downstream target gene, *AGL20*, were also the subject of research at Syngenta (WO 2007/122086). The complex nature of flowering time control, however, is likely to require research on multiple genes and modification strategies. In addition, and importantly, the approach that was followed in the current project for the first time employed the use of the SuperSAGE technology (Matsumura *et al*., 2003; 2005; 2006) to identify previously unrecognized targets for genetic modification of floral transition in beet. There were several genomic resources available for sugar beet that allowed identification of candidate genes on the basis of homology to flowering time genes from model species, and facilitated SuperSAGE data analysis and exploitation. These resources included public and proprietary EST collections and BAC libraries that were available in-house. Also, whole-genome physical mapping and sequencing of the sugar beet genome was underway or planned as part of GABI projects and was expected to allow a comprehensive annotation of the SuperSAGE data in due time.

5. Zusammenarbeit mit anderen Stellen

The project comprised key experimental work packages that were divided among the partners, subcontractors and collaborators according to the respective parties' areas of expertise as follows: Identification and molecular characterization of flowering time genes and transgenes generated in the project (CAU Kiel); genome-wide expression profiling by SuperSAGE (GenXPro as subcontractor of CAU Kiel); identification of allelic variants in candidate genes by TILLing (collaboration with the GABI-TILL consortium); sugar beet transformation with RNAi and overexpression constructs and pre-selection of transgenic events (SESVanderHave); regeneration of plants, vernalization and phenotyping for altered bolting behavior (Strube Research).

II. Eingehende Darstellung

1. der Verwendung der Zuwendung und des erzielten Ergebnisses im Einzelnen, mit Gegenüberstellung der vorgegebenen Ziele

II.1.1 Genome-wide identification and expression profiling of floral transition genes

CAU Kiel's first objective was to identify candidate genes for targeted genetic modification of flowering time in sugar beet on the basis of homology to known flowering time genes (Figure 1) and responsiveness of gene expression to vernalization. To this end, we performed tblastn-based sequence similarity searches and bidirectional best hit analyses in GenBank (http://blast.ncbi.nlm.nih.gov), the public sugar beet EST database BvGI (versions 1.0 to 3.0, http://compbio.dfci.harvard.edu/tgi), and the GABI Primary Database (http://www.gabipd.org) with flowering time control genes from *A. thaliana* and other model species. This analysis identified several putative orthologs of floral regulators in *A. thaliana* from all major regulatory pathways. In addition, work in other projects in the PI's group identified two potential key regulators of floral transition, i.e. (i) the long-sought 'bolting gene', which we termed *BvBTC1* (*BOLTING TIME CONTROL 1*; Pin *et al*., 2012), and (ii) a sugar beet homolog of the floral integrator gene *FT*. Although *BvBTC1* was first thought to be the central regulator of annuality and bolting without a requirement for vernalization, several data indicated that it may also have a regulatory function in biennials, including our observations that it has a full-length coding sequence, biennial haplotypes do not differ from annual haplotypes at evolutionarily conserved positions, and diurnal *BvBTC1* expression is similar (albeit not identical) in biennials as in annuals. *FT* is a central regulator of flowering time in *A. thaliana* and integrates signals from various floral regulatory pathways, and *ft* mutants are late-flowering and non- or only weakly responsive to vernalization (Koornneef *et al*., 1991, 1998; Kardailsky *et al*., 1999). A putative ortholog of *FT*, for which ESTs were absent from public EST collections, was identified by RT-PCR and RACE in a previous project (Eurotrans-Bio-Blossom). To further characterize this gene and facilitate a functional analysis by TILLing, we identified the full-length genomic sequence of ~12kb including ~3kb of promoter sequence, exon-intron structure, and

~2kb of the downstream intergenic region by BAC library screening and primer walking. This gene was later found to correspond to one of two *FT*-like genes recently described in sugar beet (Pin *et al.*, 2010) and will be referred to as *BvFT1* in accordance with the nomenclature by Pin and colleagues. Both *BvBTC1* and *BvFT1* were deemed prime candidates for targeted modification of flowering time in sugar beet and further analyzed in the current project. Furthermore, sugar beet homologs of the vernalization pathway genes *FLC* and *LHP1* were also selected for further analysis. *FLC* is a central repressor of floral transition in Arabidopsis and is down-regulated by vernalization, and overexpression of *FLC* leads to a delay in flowering after vernalization. Shortly before the begin of the current project, an *FLC* homolog (*BvFL1*) was isolated from beet, shown to be functionally related to *FLC* in Arabidopsis and regulated by vernalization in beet (Reeves *et al.*, 2007), but a functional analysis in beet was lacking. *LHP1* is involved in the maintenance of the inactive chromatin state of *FLC* following vernalization but also represses *FT*, and *lhp1* mutants in Arabidopsis have either early-flowering or late-flowering phenotypes depending on the genetic background of the mutants and the environmental conditions analyzed. A full-length *LHP1* homolog in sugar beet was isolated by the PI's group in the Eurotrans-Bio-Blossom project and its expression shown to be regulated by vernalization. Thus, together, the genes listed above constitute a first set of candidate genes and include the only two vernalization pathway gene homologs identified at the time in beet, *BvFL1* and *BvLHP1*, a homolog of the key floral integrator gene *FT*, and the central regulator of bolting in beet, *BvBTC1*.

In a complementary approach, floral transition candidate genes were selected on the basis of genome-wide expression profiling of vernalization response by SuperSAGE (Matsumara *et al.*, 2008). Young immature leaves harvested at various time points during a vernalization time-course experiment (Figure 2) were used to produce nine SuperSAGE libraries containing 1.7 to 2.2 $x10^6$ sequenced tags/library (giving a total of 17.4 $x10^6$ tags; Table 1). Immature (non-expanded) leaves were chosen because they were postulated by grafting (Stout, 1945; Curtis and Hornsey, 1964) and defoliation experiments (Crosthwaite and Jenkins, 1993) to be the site of vernalizing temperature perception and early events during the transition to the flowering competent phase in sugar beet. Expression data from Arabidopsis and temperate cereals suggested that key regulators of vernalization response are differentially regulated in response to vernalization. For example, the floral inducer gene *VRN1* in cereals is gradually up-regulated during prolonged periods of cold temperatures (Danyluk *et al.*, 2003; Trevaskis *et al.*, 2003; Yan *et al.*, 2003). We therefore used gradual expression changes during vernalization as indicator for a function in vernalization-dependent control of flowering. To facilitate annotation of tags and using cost-effective second generation sequencing technology (Illumina), we sequenced cDNA pooled from all nine samples and generated a database of 20106 cDNA contigs which combines publicly available sugar beet EST data and the newly generated cDNA sequences (Table 2).

Table 1. Tag counts in nine SuperSAGE libraries produced in the vernalization time-course experiment.

	Number of SuperSAGE tags per library									
Library	T_0	T_1	T_2	T_3	T_4	T_{1V}	T_{2V}	T_{3V}	T_{4V}	Total
Tags ($x10^6$)	2.01	2.23	2.12	1.74	2.17	1.74	1.85	1.77	1.78	17.41
Unique tags ($x10^3$)	54.9	54.3	50.2	56.6	57.5	54.9	57.5	56.5	55.5	67.5

Table 2. Reference cDNA sequences for tag annotation.

	Number of Illumina reads	Number of contigs	Average size of contigs [bp]	Maximal size of contigs [bp]
GABI-GENOFLOR cDNA library	75.167.775	81.127	416	3.548
Combined with public EST/TCs (MIRA assembly)	n.a.	20.106	798	3.850

Figure 3 shows the criteria for identification of tags which are significantly up-regulated in several library comparisons. Among 67515 unique tags identified by SuperSAGE, and using the p-statistics for transcript profiles devised by Audic and Claverie (1997; 99% confidence level), 490 tags are continuously upregulated during vernalization, while also being upregulated when compared to non-vernalized controls (Figure 4a; Table 3). 176 tags were identified when corresponding criteria for down-regulation of gene expression were used (Figure 4b; Table 3). We used RNAi for functional analyses and suppression of bolting in transgenic plants and had proposed to target candidate genes for floral promoter genes. We further reasoned that genes which are up-regulated by vernalization, a major stimulus of flowering in plants, are likely to include floral promoter genes, whereas genes which are down-regulated by vernalization may have a repressive effect on flowering. This assumption is consistent with expression data in the literature for the floral promoter gene *VRN1* in cereals (s. above) and the floral repressor genes *FLC* in Arabidopsis and *BvFT1* in beet, as shown recently by Pin *et al.* (2010). Thus, candidate floral promoter genes were selected among genes which are positively regulated by vernalization. Of the 490 tags which are gradually up-regulated, 169 could be assigned to a cDNA/EST contig in cDNA database generated in the current project. These contigs were annotated using BLAST and BLAST2GO (http://www.blast2go.org; Figure 5). Among these, three genes were annotated as having transcription factor activity (Figure 5). By further sequence analysis, the corresponding cDNA contigs were identified as the *FLC* homoglog *BvFL1* (Reeves et al, 2007) and homologs of other transcription factors which will be referred to as *BvTFCs*. Up-regulation of *BvFL1* is in contrast to the previously published report by Reeves *et al.* (2007) and the regulation of *FLC* in Arabidopsis, where the gene is down-regulated by vernalization, suggesting that *FLC* in *A. thaliana* and *BvFL1* may not be functionally equivalent. Further GO and sequence analysis of all cDNA contigs identified one gene which was annotated with the GO terms “nucleic acid binding” (“molecular function", level 3; Figure 5) and “regulation of transcription” (“biological process”, level 7). Intriguingly, a second vernalization-responsive gene in the same GO subcategory is *BvBTC1*, further supporting the notion that this gene may play a functional role in biennials (s. above) and may also be involved in the vernalization response. The remaining genes were assigned to a broad spectrum of molecular functional categories and included a considerable fraction of putative stress response genes including e.g. oxido-reductases and homologs of various metabolic pathway genes and enzymes without known functions in flowering time control. Finally, by using less stringent criteria for up-regulation of gene expression during vernalization, a homolog of a vernalization response gene in *Arabidopsis thaliana* was identified as an additional candidate gene. This gene is only very weakly expressed in all samples of the vernalization time course analyzed by SuperSAGE except for time point t3V (three months after begin of vernalization treatment), where it is significantly up-regulated according to a statistical analysis using the p-statistics by Audic and Claverie (1997).

In conclusion, sequence and expression analyses and literature reviews led to selection of eight candidate genes (*BvBTC1*, *BvFT1*, *BvFL1*, *BvLHP1*, *BvTFC1-4*) which were used for construction of 11 transformation vectors for functional analyses in transgenic beets (s. below). Several further genes whose expression is regulated by vernalization may also contribute to flowering time regulation but were not further considered here because of the more speculative nature of this possibility and the limited number of transgene cassettes planned in the project. According to our milestone plan for the project, it was intended to identify 15-20 *B. vulgaris* floral transition gene candidates and to select the best candidate genes on the basis of the available information for construction of 10 transgene cassettes. Thus, these project results are in good agreement with the original objectives. In further accordance with the milestone plan, the expression profiles of vernalization-responsive candidate genes identified by SuperSAGE were verified by RT-qPCR analysis.

Table 3. Numbers of tags which are differentially expressed at p ≤ 0.01 (as determined using DiscoverySpace; Robertson *et al*., 2007) during the vernalization time-course experiment.

Pairwise comparison of libraries	No. of differentially expressed tags	No. of tags up-regulated	No. of tags down-regulated
T4V vs T4	7.165	2.877	4.288
T3V vs T3	12.103	7.717	4.386
T2V vs T2	13.575	8.698	4.877
T1V vsT1	9.536	4.343	5.193
T3V vs T2V	6.988	4.110	2.878
T2V vs T1V	12.442	8.420	4.022