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Transfer of knowledge about flowering and vegetative propagation from model species to bulbous plants

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ABSTRACT The extensive characterization of plant genes and genome sequences summed to the continuous development of biotechnology tools, has played a major role in understanding biological processes in plant model species. The challenge for the near future is to generate methods and pipelines for an efficient transfer of this knowledge to economically important crops and other plant species. In the case of flower bulbs, which are economically very important for the ornamental industry, flowering time control and vegetative propagation constitute the most relevant processes for agronomical improvements. Those processes have been reasonably studied in reference species, making them excellent candidates for translational investigations in bulbous plant species. The approaches that can be taken for the transfer of biological knowledge from model to non-model species can be roughly categorized as "bottom-up" or "top-down". The former approach usually goes from individual genes to systems, also known as a "gene-by-gene" approach. It assumes conservation of molecular pathways and therefore makes use of sequence homology searches to identify candidate genes. "Top-down" methodologies go from systems to genes, and are e.g. based on large scale transcriptome profiling via heterologous microarrays or RNA sequencing, followed by the identification of associations between phenotypes, genes, and gene expression patterns and levels. In this review, examples of the various knowledge-transfer approaches are provided and pros and cons are discussed. Due to the latest developments in transgenic research and next generation sequencing and the emerging of systems biology as a matured research field, transfer of knowledge concerning flowering time and vegetative propagation capacity in bulbous species are now within sight.

KEY WORDS: *bulbous plant, flowering time control, vegetative propagation, gene regulation*

Introduction

In the last decade the establishment of full genome-sequences and the development of new biotechnology tools have dramatically increased our knowledge of plant functioning. For example, the genome sequence of *Arabidopsis* (~130 Mbp; dicot), rice (~380 Mbp; monocot) and maize (~2500 Mbp; monocot) were completed in 2000, 2002 and 2009, respectively (AGI, 2000, Schnable *et al.*, 2009, Sequencing Project International Rice, 2005). Molecular biology, genomic and transgenic research, such as loss-of-function mutagenesis and overexpression studies, have played a key role in exploiting and understanding biological and molecular functions of the thousands of genes present in the genome sequences. Nonetheless, the majority of these functional studies have been performed in plant model species, such as *Arabidopsis*, Medicago and rice. All together this provided a wealth of knowledge on the control of a large variety of biological processes and traits. Hence, the road has been paved for the implementation of this data and the transfer of knowledge from model species to relevant but less

Abbreviations used in this paper: BLAST, basic local alignment search tool; cDNA, complementary deoxyribonucleic Acid; EST, expressed sequence tag; MADS, Mcm1 agamous deficiens serum response factor; Mbp, millions of base pairs; NGS, next generation sequencing; PCR, polymerase chain reaction; RNA, ribonucleic acid; SAM, shoot apical meristem; T-DNA, transfer-DNA; VIGS, virus induced gene silencing.

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studied crop species, ultimately aiming to improve and optimize yield and quality for a sustainable agriculture.

Almost all bulbous plant species are monocots, including the economically important ornamentals tulip and lily. Bulbous plants are hardly studied at the molecular and biotechnological level and therefore this review will have a special focus on these species. Bulbous species were introduced in Western Europe in the 16th century and are nowadays primarily utilized for commercial bulb production, garden and forced fresh cut flower production and for landscape architecture. Cultivation occurs in temperate climate regions with the Netherlands being the leading producer world-wide. In total, seven species dominate the industry consisting of *Tulipa*, *Lilium*, *Narcissus*, *Gladiolus*, *Hyacinthus*, *Crocus* and *Iris* (Benschop *et al.*, 2010). Flower bulbs propagate sexually through seeds and vegetative via initiation and outgrowth of axillary meristems, which are usually located in the underground storage organ (Kamenetsky and Okubo, 2012). Like other plants, bulbs propagated from seeds undergo three developmental phases: juvenile vegetative, adult vegetative and reproductive. The duration of the juvenile vegetative phase can take several years (e.g. *Tulipa* and *Narcissus*) and only upon the transition to the adult vegetative phase, the bulb becomes competent for flower initiating signals. The vegetative phase switch from juvenile to adult depends on the physiological age, weight and size of the bulb. Subsequently, taking tulip as an example, high temperatures can induce the transition from adult vegetative to the reproductive phase, resulting in flower bud initiation. Simultaneously, dormancy is triggered and a pro-longed period of cold is required for dormancy release and internal preparation for stem elongation and flower outgrowth in the next spring. This specific life cycle is not only seen in tulip, but is common for various bulbous species, including *Tulipa*, *Crocus* and *Hyacinthus* (Kamenetsky *et al.*, 2012, Rees, 1966, Saniewski *et al.*, 2000).

In order to improve bulb productivity and ornamental characteristics, it is necessary to increase genetic variation by breeding new cultivars and potentially this can highly benefit from the implementation of biotechnological and *'omics'* tools. Currently, the development of a new tulip cultivar can take up to 20 years because of its long juvenile phase and low vegetative propagation rate (Podwyszyńska, 2005). Besides the long juvenile phase, which slows down the breeding process and the production of flowers, an agricultural problem is laid down in the precocious flower initiation by high temperatures in spring, resulting in early development of the flower bud. Consequently the flower bud is completely developed inside the bulb around harvest time, leading to either flower abortion or a decrease of flower quality in the next season because of dehydration during storage of the bulbs (Hartsema, 1961). In addition, natural vegetative propagation rates vary among flower bulbs, but on average are low due to the limited number of axillary meristems and a restriction in outgrowth of these meristems (Kamenetsky and Okubo, 2012). Together with the long juvenile phase, this makes the development of a new flower bulb cultivar a slow and time consuming process. Many efforts in understanding and improving the physiological nature of flowering and vegetative propagation in bulbous plants took place in the last decades (Aung and Hertogh, 1979, Balk and de Boer, 1999, Beijer, 1952, Lambrechts *et al.*, 1994, Rietveld *et al.*, 2000); however, the majority of these studies focused on physiological factors and limited molecular and genomic studies have been performed. Although various reasons can be brought forward for this, the large genome sizes for bulbous plants (Tulip ~25000 Mbp; Lily ~36000 Mbp) and technical difficulties in isolating e.g. RNA from bulb scales have been particularly decisive in this (Shahin *et al.*, 2012).

Here, we will briefly summarize the current knowledge on flowering time control and vegetative propagation gained from studies in model plant species, since these are the two most important biological processes for agronomical improvements of bulbous plant species cultivation. Subsequently, we will give an overview of approaches to transfer this type of knowledge from model plants to crop species and how transgenic and *'omics'* technologies can be supportive. Various examples will be given from studies that used such a strategy, including an overview of the technologies that are relevant for bulbous plant species. In the final concluding section a prospect will be given how novel emerging technologies,

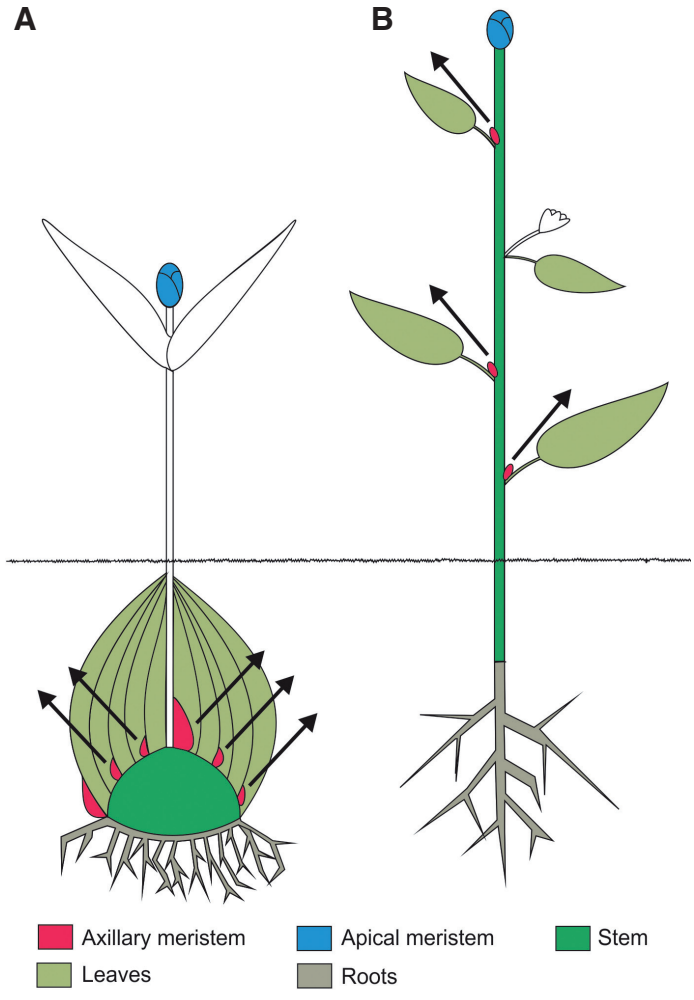

Fig. 1. Architecture of a bulbous and a non-bulbous plant. (A) *Tulip,* **(B)** *model dicot plant. Initiation of axillary meristems takes place in the axils of bulb-scales* **(A)** *or leaves* **(B).** *They form a bud like structure and undergo a period of dormancy. Once bud dormancy is broken, axillary buds grow out and develop into daughter bulbs in bulbous plants, or axillary branches in a typical dicot plant. In tulip, normally only two of the axillary buds will develop into daughter bulbs and once the apical bud blooms and dies, the closest axillary bud will become the apical bud for the next season. In bulbous plants the stem is called basal plate and it is a modified stem; Bulb-scales of bulbous plants are modified leaves. Arrows represent axillary bud outgrowth.*

bioinformatics, and systems biology can increase the efficiency and strength of this type of research and move the field from gene-by-gene approaches into a comprehensive genome-wide level.

What is known about flowering and vegetative propagation from model systems

Although the best studied model system, *Arabidopsis*, is a dicot, and the majority of bulbous plant species are monocots, the regulatory mechanisms underlying important agricultural traits appeared to be conserved in various cases. Hence, knowledge gained in *Arabidopsis* can be informative for studies in bulbous plants. Strong conservation between *Arabidopsis* and the monocot rice was observed e.g. for the genes involved in the photoperiod flowering time pathway (Izawa *et al*., 2003). The same holds for various hormonal signalling components and the key transcription factors involved in axillary meristem formation and outgrowth, which is directly related to vegetative propagation capacity in bulbous species (Finlayson, 2007, Kebrom *et al*., 2013). Nevertheless, various exceptions are known and in general best results are obtained when using a closely related model species as starting point. Therefore, we will discuss mainly knowledge gained from *Arabidopsis*, but when relevant, complemented with information from other dicots and monocot species.

Vegetative propagation

Shoot branching is a vegetative process determined by axillary meristems and it determines the architecture, biomass and reproductive success of a plant. Initiation of an axillary meristem results in the formation of a bud that will undergo a period of dormancy. Once the right environmental or endogenous plant factors release the bud from dormancy, it will grow and develop into a branch or a propagule in the case of flower bulbs (e.g. daughter bulb, bulblet, bulbil), a process known as bud outgrowth. Hence, the processes of axillary bud initiation and axillary bud outgrowth together determine the vegetative propagation rate in bulbous species (Fig. 1).

Several genes promoting axillary bud initiation have been identified in different model species (Bennett and Leyser, 2006, Kebrom *et al*., 2013) and their supposed functions could be confirmed by transgenic approaches. For instance, a transcription factor of the GRAS family characterized in tomato, rice and *Arabidopsis*, - Lateral suppressor (*Ls), Monoculm1 (MOC1)* and *LATERAL SUPPRESSOR* (*LAS),* respectively - is responsible for the establishment of an axil identity and maintenance of meristematic capacity via prevention of cell de-differentiation (Bennett and Leyser, 2006, Greb *et al*., 2003, Li *et al*., 2003, Schmitz and Theres, 2005, Ward and Leyser, 2004). A second key regulatory gene discovered in tomato, *BLIND (Bl)*, encodes a MYB transcription factor that also promotes axillary bud initiation but its function is independent of *Ls*. The *Bl* ortholog in *Arabidopsis* is *REGULATOR OF AXILLARY MERISTEMS1 (RAX1)* (Keller *et al*., 2006, Müller *et al*., 2006). A third regulator identified in *Arabidopsis*, *REGULATOR OF AXILLARY MERISTEM FORMATION (ROX)* has orthologs in rice *LAX PANICLE1 (LAX1)* and maize *Barren stalk1 (Ba1)*, although the latter two also affect inflorescence branching (Yang *et al*., 2012). During vegetative development in *Arabidopsis*, *LAS* and *RAX1* influence the expression of *ROX* and axillary bud initiation occurs when *ROX* expression ceases (Yang *et al*., 2012). In contrast, *LAX1* transcripts in rice are detected only after the axillary bud has initiated (Oikawa and Kyozuka, 2009), suggesting that the molecular control of *ROX*-like genes may differ in timing between monocots and dicots.

Occurrence of bud outgrowth depends on the factors that release buds from dormancy. Apical dominance, which is the ability of the shoot apex of the plant to prevent outgrowth of axillary meristems, and therefore branching, is one of the most studied phenomena controlling dormancy in axillary buds. This control is mediated by a balanced hormonal signalling between auxin, cytokinin and the recently discovered strigolactones (Kebrom *et al*., 2013). Evidence for a role of strigolactones in axillary bud outgrowth is given by *ramosus (rms)* mutants in pea, *decreased apical dominance (dad)* in petunia, *more axillary growth (max)* in *Arabidopsis,* and *dwarf* (*d*) or *high tillering dwarf* (*hdt*) in rice (Booker *et al*., 2005, Ishikawa *et al*., 2005, Liu *et al*., 2009, Morris *et al*., 2001, Napoli, 1996) In *Arabidopsis* MAX1, MAX3 and MAX4 are involved in strigolactone biosynthesis while MAX2 plays a role in strigolactone signalling. Although the exact crosstalk between auxin, strigolactones and cytokinins in the control of shoot branching is not yet entirely understood, it is clear that auxin and strigolactones inhibit bud outgrowth while cytokinins promote it. In this system, a bud-specific gene that promotes bud arrest could be the key element to integrate the bud outgrowth pathway. Indeed, such a gene exist and is represented by *Teosinte branched1 (TB1)* in maize and *BRANCHED (BRC1)* in *Arabidopsis* (dicot). *TB1* was first identified in maize and appears to encode for a transcription factor from the TCP family (Aguilar-Martínez *et al*., 2007). Evidence in *Arabidopsis* and pea show that the *TB1* ortholog *BRC1* is up-regulated by strigolactones and down-regulated by cytokinins (Aguilar-Martínez *et al*., 2007, Braun *et al*., 2012). A more recent study supports the idea of BRC1 as a second messenger to induce and maintain bud arrest by negatively regulation of cell cycle, ribosome translation, and promotion of Abscisic Acid (ABA) signalling (González-Grandío *et al*., 2013). Because, outgrowth of axillary buds seems to be the major limiting factor in vegetative propagation of bulbs, the strigolactone signalling pathway and *TB1*-like genes are first targets of choice to study and optimize vegetative propagation in these plant species.

Flowering time control and flowering induction

Besides branching and axillary bud development, flowering time is an important trait influencing reproduction capacity in bulbous species. Plants are continuously sensing their environment, for being in the reproductive phase under optimal conditions and securing their reproductive success. Besides environmental cues, such as photoperiod and temperature, flowering time is also controlled by endogenous signals, including hormone levels and plant age (Lang, 1952). In the model plant *Arabidopsis* the vegetative phase transition and floral induction, are well studied at the molecular level and the complex gene regulatory networks underlying these processes have recently been reviewed (Andres and Coupland, 2012, Srikanth and Schmid, 2011). We will discuss flowering time control here only briefly, with a focus on the pathways that are the most important for flowering in most of the bulbous species (Fig. 2), which are the aging and temperature pathways. The juvenile vegetative phase (aging pathway) can take up to seven years in bulbous species. Upon reaching the adult vegetative stage, the transition to reproductive development can be induced, which in tulip e.g. is triggered by relative warm temperatures in the spring or early summer (ambient temperature pathway). However, for

development of the floral meristem into a complete flower and for elongation of the floral stem, a prolonged period of cold is essential (dormancy release), in analogy with bud dormancy release in trees (Cooke *et al.*, 2012).

Plant age is one of the endogenous factors that can be linked with developmental phase transitions and competence of the shoot apical meristem for environmental signals triggering flowering. The age-dependent vegetative transition in *Arabidopsis* is regulated by *microRNA156* (*miR156*) and the *SQUAMOSA PROMOTOR BINDING PROTEIN-LIKE* (*SPL*) genes that are targeted by this microRNA. The repression of *miR156* results in up-regulation of several *SPL* genes which promotes vegetative transition (Fornara and Coupland, 2009). Two recently published studies showed that *miR156* levels are responding to sugars (Yang *et al.*, 2013, Yu *et al.*, 2013). Whereas a bulb is a storage organ and it is well known that sugars get re-located towards the shoot apical meristem and stem (sinks) upon flowering-inducing temperature changes (Lambrechts *et al.*, 1994), it will be of interest to focus on this particular pathway in the hunt for signalling components involved in flowering time control of bulbous species.

After the switch from the juvenile to the adult vegetative phase, the plant becomes competent for flowering inducing external cues. Furthermore, reproductive development is triggered by the activation of *microRNA172* (*miR172*) by the *SPL* genes, which results in the repression of a set of *APETALA2 (AP2)*-like genes, acting as repressors of flowering (Zhu and Helliwell, 2011). Both microRNAs *miR156* and *miR172* are conserved in dicots and monocots (Axtell *et al.*, 2007). Although, the age dependent phase transition is studied to a lesser extent in monocots (Strable *et al.*, 2008, Tanaka *et al.*, 2011) (Fig. 2), performed experiments reveal a high level of conservation in the regulatory mechanisms controlling flowering time in between different species.

Vernalization is the requirement for a period of prolonged cold to overcome a block on flowering in winter annual plants. In *Arabidopsis FLOWERING LOCUS C* (*FLC*) is the key floral repressor in this process, and this transcription factor was shown to act as a direct transcriptional repressor of the so-called floral integrator genes *FT (FLOWERING LOCUS T)* and *SUPPRESSION OF OVEREXPRESSION OF CONSTANS1* (*SOC1*) (Fig. 2). *FLC* is activated by the positive regulator *FRIGIDA* (*FRI*) that acts in a large multi-protein complex. During winter, the transcriptional regulator VERNALIZATION INSENSITIVE3 (VIN3) will respond to a prolonged period of cold, resulting in its gradual activation. As a consequence *FLC* will be repressed providing the shoot apical

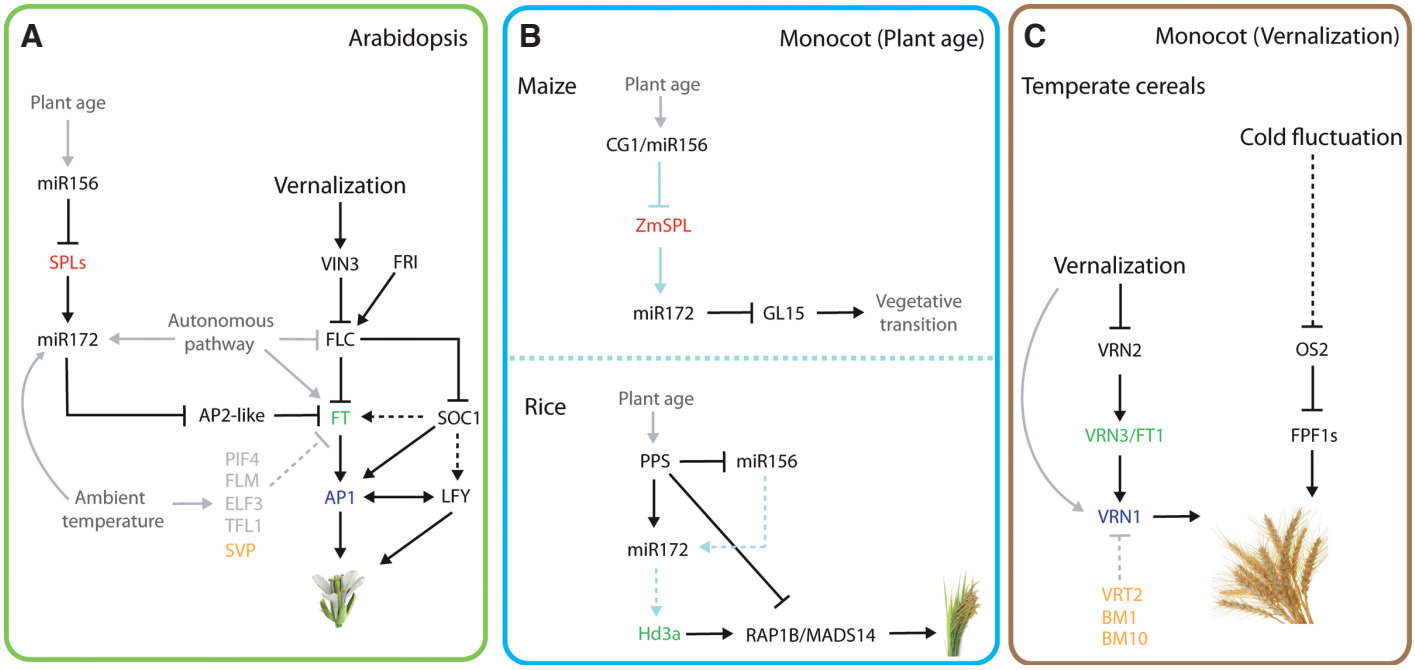

Fig. 2. Comparison of the gene regulatory networks for flowering time control in dicots and monocots. *In* Arabidopsis **(A)** *upon aging miR156 is repressed leading to the up-regulation of selected SPL genes which promote the vegetative phase transition (aging pathway). External cues, e.g. ambient temperature, trigger the activation of miR172 via the SPL genes leading to the repression of the floral repressors AP2 and AP2-like genes. In order to be able to flower, winter annual* Arabidopsis *ecotypes first needs a pro-longed period of cold leading to the activation of VIN3 and repression of FLC. Ultimately, this results in the activation of the floral integrators FT and SOC1, followed by the activation of the floral meristem identity genes AP1 and LFY. In the monocot maize* **(B, top)**, *the vegetative phase transition is regulated by the suppression of the AP2-like gene GLOSSY15 (GL15) through the activation of miR172. CORNGRASS1 (CG1) encodes miR156 and similar to* Arabidopsis, *might represses ZmSPL leading to the activation of miR172. In the monocot rice* **(B, bottom)**, *PETER PAN SYNDROME (PPS) is involved in the repression of miR156 and the activation of miR172. This might occur directly by PPS or indirectly (dotted blue arrow) through miR156. Upon unfavourable environmental conditions, PPS represses RAP1B/MADS14, independent of Hd3a (rice FT homolog). In monocot temperature cereals* **(C)** *the FT homolog VRN3/FT activates VRN1 upon a prolonged period of cold, leading to flowering. Nevertheless, the SVP homolog VRT2 represses VRN1. Shorter periods of cold repress OS2 which inhibits stem elongation through FPF1s. Taking into account this knowledge from model species and assuming general conservation of the gene regulatory networks, a putative flowering controlling network can be designed for bulbous species. Genes with similar kind of functions in the different species are marked with the same colour.*

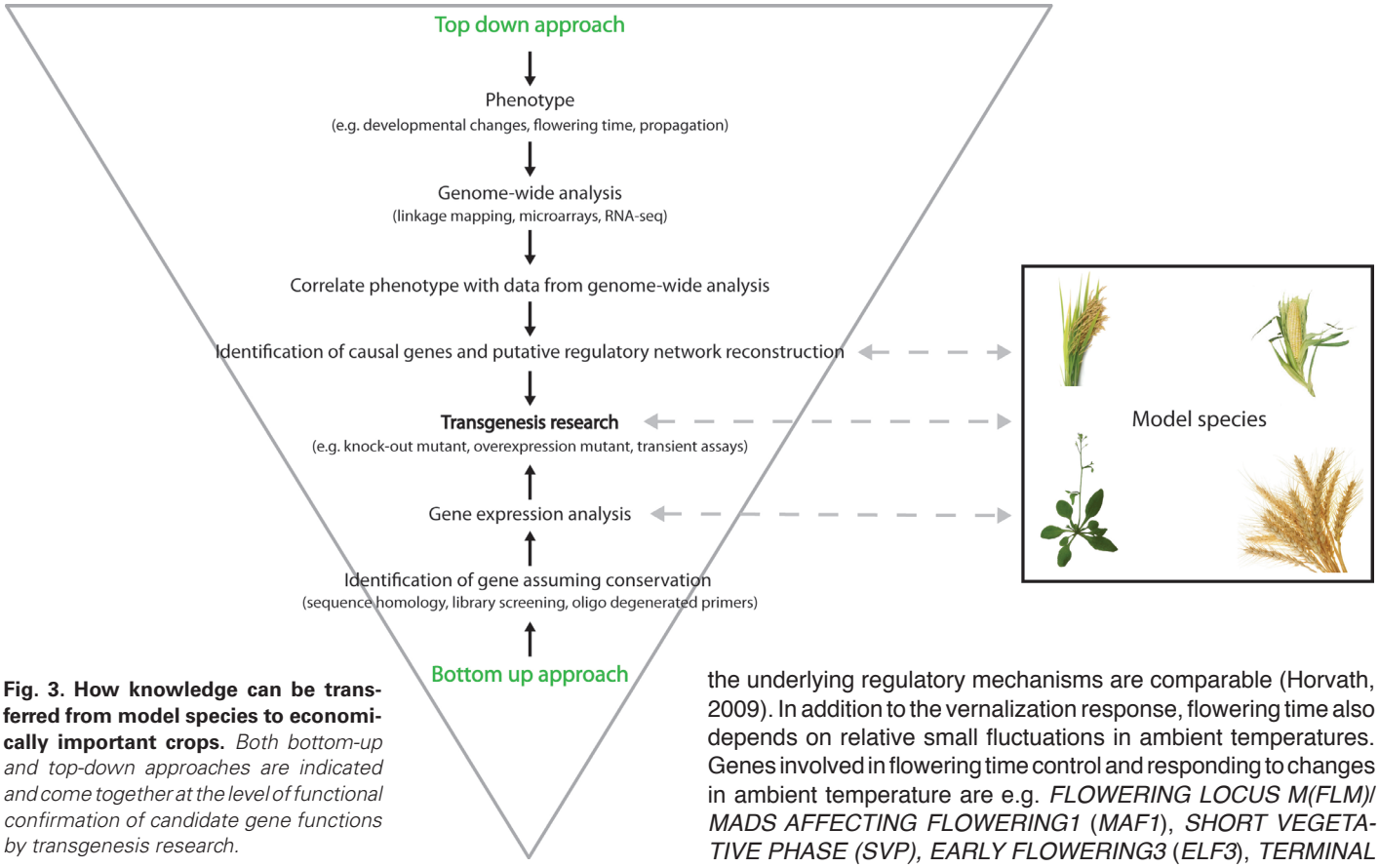

Fig. 3. How knowledge can be transferred from model species to economically important crops. *Both bottom-up and top-down approaches are indicated and come together at the level of functional confirmation of candidate gene functions by transgenesis research.*

meristem competence for floral inducing cues, such as optimal temperatures and appropriate photoperiod conditions (Choi *et al.*, 2011). In monocots however, *FLC*-like genes could not been identified. In wheat a different gene, *VERNALIZATION2* (*VRN2*), encoding for a Zinc finger-CCT domain containing transcription factor (Yan *et al.*, 2004), is down-regulated by vernalization. This repression results in the activation of the *FT* homolog *VERNALIZATION3* (*VRN3*)/*FLOWERING LOCUS T1* (*FT1*) and the *APETALA1*-like *VERNALIZATION1* (*VRN1*) gene during a period of prolonged cold (Alonso-Peral *et al.*, 2011, Yan *et al.*, 2006). Three genes homologous to the *Arabidopsis SVP* gene; *VRT2*, *BM1* and *BM10* respectively, are able to repress *VRN1* but their role in vernalization or floral transition is not completely understood (Kane *et al.*, 2005, Trevaskis *et al.*, 2007). Besides a pro-longed period of cold (vernalization response), short cold stresses repress the grass specific MADS box gene *ODDSOC2* (*OS2*). A proposition was made that *OS2* is present in a pathway that delays the transition to reproductive development and that additionally inhibits stem elongation (Greenup *et al.*, 2010). Altogether, this suggests that the vernalization response has evolved independently in monocot and dicot plants, although members from the MADS box transcription factor family play an important role in both. Bulbous plants, such as tulip, also require a prolonged period of cold. Though, in this case it is not essential for the meristematic switch from vegetative to reproductive development, but to release dormancy in the already existing floral bud and to induce stretching of the floral stem. Despite that this dormancy release is different from the vernalization response, more and more evidence is provided that the underlying regulatory mechanisms are comparable (Horvath, 2009). In addition to the vernalization response, flowering time also depends on relative small fluctuations in ambient temperatures. Genes involved in flowering time control and responding to changes in ambient temperature are e.g. *FLOWERING LOCUS M(FLM)/MADS AFFECTING FLOWERING1* (*MAF1*), *SHORT VEGETATIVE PHASE (SVP), EARLY FLOWERING3* (*ELF3*), *TERMINAL FLOWER1* (*TFL1*) and *PHYTOCHROME INTERACTING FACTOR4* (*PIF4*) (Balasubramanian and Weigel, 2006, Kumar *et al.*, 2012; Lee *et al.,* 2013; Posé *et al.,* 2013). Nevertheless, in contrast to the wealth of knowledge on the vernalization pathway, insight in the gene regulatory network underlying the ambient temperature pathway is just emerging.

Ways to transfer knowledge from model plants to economically important crop species

To transfer the wealth of knowledge gained from studies in model species towards crops and e.g. bulbous plant species, diverse roads can be taken. According to the methodology used to link the molecular basis of life (e.g. genes) with biological functions, such methods can be divided in bottom-up or top-down approaches (Fig. 3). The former one uses deductive reasoning, meaning that the knowledge is built from the constitutive parts (e.g. genes) to the systems, while top-down requires inductive reasoning: from systems to causal genes.

Bottom-up approach

The start point of this approach is the identification of putative orthologues genes in crops for genes of interest in model species (Salentijn *et al.*, 2007). In general this is based on sequence homology and the assumption that the molecular pathways underlying the control of the biological processes, and hence the involved genes, are conserved. A widely used method in the past was the identification of highly homologous genes by genomic or cDNA library screenings (e.g. (Sun *et al.*, 1999, Xu *et al.*, 1995)). For

this purpose hybridization can be applied or alternatively PCR-based methods, using degenerated oligonucleotides. Once the unknown target genes are identified, they can be sequenced and subsequently compared *in silico* with the gene sequences from the model species. An advantage of this method is that construction of such libraries does not require *a priori* genome sequence information. However, to date there are only limited comprehensive genomic libraries available for flower bulb species, likely due to the complex genome sizes. When sequence information is available for the species of interest, identification of homologues is normally done *in silico* via BLAST-based sequence alignments (Altschul *et al.*, 1990). However, there are limitations to the above discussed simplistic approaches, given by the fact that sequence similarity does not always imply functional similarity. This is nicely exemplified by differences in function for key genes in the vernalization pathway between monocots and dicots (e.g. *AP1*-like genes; Fig. 2). Furthermore, large-scale evolutionary events such as duplications can cause functional divergence for paralogues genes. When evolutionary events are taken into account, comparative studies, such as synteny mapping can provide information on orthology of the blasted sequences (McCouch, 2001). Whereas in the past, this was restricted to species for which the genome was sequenced or for which a detailed genetic map was available, integrating high-throughput Next Generation Sequencing (NGS) data makes it possible to apply this type of studies to crops that lack a reference genome sequence (Galvão *et al.*, 2012) and hence, make it also possible to use synteny mapping for bulbous plant species in the near future. Regardless whether orthology will be taken into account, various experimental tools can be applied to guide the identification of genes or proteins with identical functions based on intrinsic characteristics of the molecules, such as protein-protein interaction capacity or their specific expression patterns.

Top-down approach

Top-down methodologies build vast amounts of high-throughput data in order to establish systems from which identifying causal genes would be feasible (Fig. 3). Large scale phenotyping platforms coupled to linkage mapping, and gene expression-based analyses, such as the generation of Expressed Sequenced Tags (EST) or genome-wide transcriptome profiling via microarray analyses or RNA-seq, are examples of sources for such large-scale data sets. EST datasets are a rich source for designing custom-made DNA microarrays (Lorenz *et al.*, 2003), but for many species of interest there are no sufficient datasets available to create a proper microarray platform. In that case, cross-species microarrays, in which probe sequences are derived from a model species and hybridization is performed with material from a crop of interest, is an attractive alternative to profile expression patterns (e.g. (Moore *et al.*, 2005, Wang *et al.*, 2010)). However results have to be interpreted carefully because of variance in efficiency of probe-transcript hybridization, caused by differences in sequence similarities or e.g. number of gene copies, due to species-specific duplication events (Lu *et al.*, 2009). Unlike classical microarray experiments, RNA-seq does not require genome sequence information (Wang *et al.,* 2009), neither *a priori* knowledge of gene functions. Furthermore, the method is highly sensitive and accurate providing detailed insight in gene transcription levels, as well as splicing variants across different physiological or morphological samples. Together, these characteristics make this technology an ideal tool to gain insight in the transcriptome of bulbous plants and to study differential gene expression for relevant biological process in these species. Nevertheless, assembling the enormous amount of short reads produced by RNA-seq is a bioinformatic challenge (Martin and Wang, 2011); especially for crops that lack a reference genome, which is the case for many economically important crops and in particular for bulbous plants. In absence of a reference genome, *de novo* transcriptome assembly is used as first approach (Garber *et al.*, 2011). A successful example of the latter approach, was recently presented for grapes, that like bulbous species preferably sustain through vegetative propagation (Venturini *et al.*, 2013). Besides transcriptomics data, information from other *"omics"* types of approaches can be implemented. Currently, after transcriptomics the proteomics field is the most advanced and detailed quantitative information can be obtained at the protein level (Bindschedler and Cramer, 2011, Kaufmann *et al.*, 2011). Also metabolomics is improving, but generated datasets are more fragmented and improvements of both throughput and reproducibility are needed (Saito and Matsuda, 2010).

The next step for all above mentioned top-down approaches, aiming to obtain information on gene activity and intrinsic gene product characteristics at a genome-wide scale, is the identification of genes or sets of genes that behave in a manner associated to the biological process of interest. Subsequently, potential gene regulatory networks can be reconstructed based on this information, which can be compared to and fed back to knowledge from model species (Fig. 3). In this respect it is good to realize that for the usage of e.g. metabolomics data an additional hurdle needs to be taken in correlating metabolite concentrations to e.g. gene expression patterns and finally gene functions.

Verification of gene function

Both bottom-up and top-down approaches give a selection of genes that are potential key players in the biological process under study, and for which preferably the function should be validated. In *Arabidopsis* this is usually done through the selection of loss-of-function mutations in collections of T-DNA insertion plants (Slater *et al.*, 2003). Alternatively, stable transformants can be generated or functions can be investigated based on transient expression assays by agro-infiltration or virus induced gene silencing (VIGS) (Lu *et al.*, 2003, Yang *et al.*, 2000). The majority of methods that are available today for gene function verification depend on transgenic approaches. Despite that these technologies are already available for thirty years and have undergone various improvements over the last decades, it is still far from trivial to transform any desired plant species. Therefore, it is still common practise to perform gene function verifications by overexpression or complementation studies using a model species as target (cross-species analysis) (Li *et al.*, 2013, Tsaftaris *et al.*, 2012).

Examples of successful knowledge transfer to bulbous plants

Bottom-up "gene-by-gene" approach

Several of the above discussed methods to transfer knowledge from model species to crops have been used already in bulbous species. Probably one of the best known examples of the bottom-up approach is related to the specification of floral organ identities by MADS box transcription factor genes according to the ABC-model (Ferrario *et al.*, 2004, Litt and Kramer, 2010, Rijpkema *et al.*, 2010).

Floral organs in higher eudicots are organized in four concentric whorls, with sepals in the outer whorl, petals in whorl two, stamens in whorl three and carpels in the inner fourth whorl. The classical ABC model predicts the establishment of the four floral organ identities by the combinatorial action of MADS domain transcription factors and the accessory gene regulatory network appeared to be highly conserved. Based on the assumption that this network will also be conserved in bulbous flowers, hypotheses were generated to explain particular flower mutants in these species. Classical examples are the so called 'double flowers', in which stamens are converted into petals or petaloid organs, which in theory can be caused by alterations in B- or C-class MADS box genes. Expression studies in the double-flowered lily "Elodie" provided evidence that this phenotype indeed was caused by the miss-expression of the putative Lily C-class gene *LelAG1* (Akita *et al.*, 2008). Besides the C class gene, a putative A class (*AP1*-like) and other MADS box genes of the C/D class have been identified in *Lilium longiflorum* (Chen *et al.*, 2008, Tzeng and Yang, 2001). Also in *Crocus sativus* a putative *AP1* gene was identified as well as a *SEPALLATA3* (*SEP3*)–like gene from the E-class (Tsaftaris *et al.*, 2011, Tsaftaris *et al.*, 2004). Despite strong conservations in flower organisation, plants belonging to the *Liliaceae* family have in general a slightly modified flower structure with two almost identical outer floral whorls, known as tepals. Based on this phenomenon a modified ABC model was proposed (van Tunen *et al.*, 1993), suggesting that class B genes are also expressed in whorl one, leading to the same petaloid identity in the outer two whorls. The putative class B genes from *Tulipa gesneriana* were cloned and characterized (Kanno *et al.*, 2003). In agreement with the hypothesized alternative model, the two *DEFICIENS* (*DEF*)–like genes *TGDEFA* and *TGDEFB* as well as one *GLOBOSA* (*GLO*)–like B-type gene *TGGLO,* were found to be all expressed in whorls one, two and three. The same model is also supported by the identification and analysis of B-class floral homeotic gene *PISTILLATA* (*PI*)/*GLO* in *Crocus sativus* (Kalivas *et al.*, 2007). All together, these examples show the power of a "gene-by-gene" bottom-up approach in case of well-studied and strongly conserved biological processes.

Top-down "transcriptome profiling" approach

Performing large-scale expression studies coupled to phenotyping is an advanced technology to identify key genes involved in a particular biological process. In lily e.g., a custom-made cDNA microarray was designed and generated, consisting of several cDNA's obtained from different pollen-related tissues (Huang *et al.*, 2006). Following, a differentially expressed gene was identified encoding for a putative protein containing ankyrin repeats and a RING zinc-finger domain, named *LIANK*. Comparison of *LIANK* to functionally characterized genes in model plants suggested ubiquitin ligase activity for the gene product. Further experiments could confirm this molecular function and revealed an important role for this gene in polar pollen tube growth, showing the relevance of the followed approach. Despite the potential of this method and the large number of examples of success stories in a variety of food crops, the approach has been hardly explored in bulbous plant species.

Gene function verification using model species

Upon the identification of functional analogues genes, verification of the function is an important process. Monocots are known to be recalcitrant to *Agrobacterium*-mediated transformation and therefore most of the flower bulb transformations have been achieved through gene-gun techniques (e.g. (De Villiers *et al.*, 2000, Kamo *et al.*, 1995, Watad *et al.*, 1998)). However, a major drawback of gene-gun transformation over *Agrobacterium*-mediated transformation is the lack of stable integrations on one hand and the unintended, but frequently observed integration of multiple gene copies in the case of a successful integration on the other hand. The latter can be a trigger for undesirable recombination events, genomic rearrangement, or silencing of the transgene (Hooykaas and Schilperoort, 1992). Conveniently, evidence has been provided for the presence of certain *Agrobacterium* strains being able to infect flower bulb species such as *Ornithogalum* (Van Emmenes *et al.*, 2008), *Gladiolus* (Kamo *et al.*, 1995) and *Lilium* (Cohen and Meredith, 1992). More recently Li and collaborators proved that insertion and stable integration of *Zm401* gene in *Lilium* is possible via *Agrobacterium*-mediated transformation, which opens the door for more transgenic efforts in flower bulbs (Li *et al.*, 2008). Nevertheless, in general transformation of bulbous plants is tedious and stable transformation frequencies are low (Lu *et al.*, 2007, Wang *et al.*, 2012). Therefore, heterologous complementation studies in *Arabidopsis* are widely used as an alternative to verify the function of a candidate gene found in bulbous species. For example, a homolog of *CENTRORADIALIS* (*CEN*)/*TERMINAL FLOWER* (*TFL*), *CsatCEN/TFL1* respectively, was cloned from *Crocus sativus* and functionally characterized in *Arabidopsis*. In *Arabidopsis*, *TFL* controls axillary meristem identity, inflorescence development and flowering time (Alvarez *et al.*, 1992). Overexpression of *CsatCEN/TFL1* in a *tfl1 Arabidopsis* mutant background resulted in complementation of the mutant phenotype, indicating that the gene isolated from *C. sativus* is able to function as *TFL1* (Tsaftaris *et al.*, 2012). A similar study revealed that a *FT*-like gene in *Narcissus tazetta* var. *chinensis*, known as *NFT1*, act as a flowering time regulator when ectopically and constitutively expressed in *ft-3* mutant *Arabidopsis* plants. In these transgenic lines, *SOC1* a target of *FT* showed to be up-regulated as expected based on FT functioning in *Arabidopsis* (Li *et al.,* 2013).

Besides stable transformation, transient technologies, such as VIGS, have been applied in bulbous species. A fragment of a putative *PDS* gene supposed to encoding phytoene desaturase, which is involved in carotenoid metabolism and photosynthesis, has e.g. been derived from lily and caused a bleaching phenotype in *N. benthamiana* after infiltration (VIGS). This phenotype was expected, because it is know that silencing of *PDS* results in photo bleaching symptoms caused by a decrease in leaf carotene. This reveals that genes of monocot species can be used to silence their counterparts in the dicot *N. benthamiana* regardless of their distant evolutionary relationship (Benedito *et al.*, 2004, Wang *et al.*, 2009) and providing hints for possible functions of the used genes.

Although the above mentioned examples show the success and power of heterologous functional analyses based on stable or transient transformation, it is good to realize that these type of experiments do in principle not indicate more than that a gene from a crop has sufficient sequence homology and overlap in functional domains to take over the activity of the endogenous gene in the model system. Consequently, this is no guarantee that a similar function can be assigned to the identified gene in the crop species. Difference in the spatial or temporal expression pattern might already withhold the gene from its supposed function based on the heterologous functional analysis.

Future directions and challenges

So far most molecular-oriented research studies in recalcitrant crops and bulbous plants have focussed on the identification of a single candidate gene. Analyses of complete regulatory pathways, as is nowadays common in model species, are hardly done yet. However, with the speed NGS technologies are developing (Schneeberger and Weigel, 2011), molecular technologies become attractive tools to analyse important biological processes in non-model species. Shahin and collegues (2012) provided e.g. the first transcriptome dataset of lily and tulip by sequencing of ESTs with the 454 NGS technology (Roche; http://www.454.com/). Comparative genomics helped with the search for gene conservation between tulip and lily, and the contigs could be annotated on the basis of the rice genome annotation (Sequencing Project International Rice, 2005). Subsequently, molecular function, biological process and cell component were predicted for the identified genes that all together resemble about 40% of the lily and tulip transcriptome. Hence, this approach provides fast insight in the active part of bulbous plants genomes, with a limited investment and avoiding the need for deciphering the complete genome sequence, which in the case of tulip is 200 times the size of the *Arabidopsis* genome. Although this is a great step forward, the authors realized and emphasized that deeper sequencing and analysis of time series for various tissues or cell types is essential to obtain sufficient information for extended comparative and functional gene studies. Furthermore, traditional sequencing techniques were producing long contiguous DNA sequence reads up to 1 kb in length; however, the majority of the latest introduced NGS platforms generate huge quantities of short sequence tags (50 to 100 bp), requiring sophisticated assembly algorithms and bioinformatics solutions (Reviewed in: (Nagarajan and Pop, 2013). Besides tackling this problem by a bioinformatic approach, technical improvements such as paired-end sequencing, helps to solve the assembly problem. Additionally, output from different platforms (e.g. PacBio; http://www.pacificbiosciences.com/) can be incorporated to overcome this problem to a certain extend. Nevertheless, the biggest barrier in this type of research will not be the generation of large scale data sets and the identification of complete gene sequences, but to extract the genes and alleles of importance for the process under study; or in other words, to find the needle in the haystack. In this respect it is good to take into account that the success rate of RNAseq experiments for gaining knowledge in a particular biological process strongly depends on a well-defined research question, followed by detailed temporal and spatial differential expression analyses (Van Verk *et al.*, 2013). In addition to the correct input of biological material and the usage of optimal algorithms to extract genome-wide differential gene expression patterns, it is of utmost importance to improve the methods for the annotation of the identified genes. As discussed above, simple blast-based alignments are a good starting point, but in the case when no or only low homology exist with known gene sequences, other technologies are essential. Recently, bioinformatics and systems biology tools have been developed for this purpose, in which e.g. domain co-occurrence networks are generated (Wang *et al.*, 2013) or information from various data sources or prediction programs is combined (Kourmpetis *et al.*, 2011).

Despite the importance of bulbous plants for the ornamental industry, these species remained under investigated at the genetic and molecular level. However, thanks to the latest developments in transgenic research, the *"omics"* area, and in the field of systems biology, the detailed study of flowering and vegetative propagation in bulbous plants, resembling the two most important biological processes for agronomical improvements, comes in sight. In a breeders perspective, shortening of the juvenile phase will help increasing the speed of selection processes for new varieties, with e.g. improved bulb productivity, ornamental characteristics and pathogen resistance. Hopefully, these developments will keep this sector flourishing in the coming century.

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