OPINION

PHOTOPERIODIC FLORAL INDUCTION AND BIOLOGICAL CLOCK : CROSS-TALKS BETWEEN INDEPENDENT OBSERVATIONS

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I recently read a paper of Heintzen *et al.* published in the *Proceedings of the National Academy of Sciences of the U.S.A.* (8) and entitled : '*At*GRP7, a nuclear RNA-binding protein as a component of a circadian-regulated negative feedback loop in *Arabidopsis thaliana*'. Some readers of the *Flowering Newsletter* may have missed it, since nothing in the title reveals that the subject of this paper may have some relation to flowering. *AtGRP7* is the *Arabidopsis* homologue of *SaGRP1*, a gene that was picked up in an attempt to clone sequences differently expressed during floral induction in the long-day plant (LDP) *Sinapis alba*. Going back to the original research on *Sinapis* (6,7), cross-talks arose in my mind forcing me to jump from one paper to another and this note emerged from the turmoil.

As part of an attempt to analyse the molecular basis of rhythmic phenomena, Heintzen *et al.* searched for transcripts that are preferentially expressed around Zeitgeber time[1] ZT16 (i.e. 16 h after the onset of the light phase) in *Sinapis alba* (6). Since this time point coincides with maximal sensitivity of the plants to night breaks (9), the rationale was that circadian-controlled activities peaking at that time might be associated with photoperiodic floral induction. A cDNA library was constructed from the upper half of *Sinapis* plants - including leaves, stem and apex - harvested at the end of a 16-h LD (ZT16). Before cloning, the cDNA was subtracted with poly(A)$^+$RNA extracted from plants sampled at ZT4 (middle of an 8-h short day (SD)). The library was then differentially screened with cDNA probes derived from plants harvested at ZT16 or ZT4. Both probes were enriched against each other. Clones that hybridised more strongly with the ZT16 probe turned out to be one category showing homology to Germin Like Proteins, thus named *Sa*GLP (6), while those that hybridised preferentially with the ZT4 probe

[1] The environmental signals that are used by the clock for entrainment are called 'Zeitgebers' ('time givers'). Since a common Zeitgeber in plants is the onset of illumination, time is expressed in hours from the beginning of the light phase.

felt into 2 sub-groups encoding Glycin Rich Proteins, *Sa*GRP1 and *Sa*GRP2 (7).

The *SaGLP* and *SaGRP*s genes show cyclic expression : in 8-h SD, the relative amount of *GRP*s transcripts peaks between ZT8 and ZT12 while those of *GLP* show a maximum around ZT12 to ZT16. These fluctuations persist when plants are transferred to continuous light (LL) or continuous darkness (DD), though the mRNA levels undergo dramatic damping in DD. Thus, these genes show circadian regulation. Interestingly, the abundance of *SaGRP*s and *SaGLP* transcripts is not modified by a change in daylength (6,7), suggesting that they were identified during the screening procedure due to their large daily fluctuation and not their photoperiodic control. It is also noteworthy that neither GLP nor GRPs proteins undergo detectable fluctuations, possibly because of their accumulation.

SaGLP was shown to encode a 22kDa protein predominantly associated with primary cell walls in the epidermis and spongy parenchyma of young leaves (6). Last year, the same kind of protein (*Pn*GLP) was associated with photoperiodic floral induction in the cotyledons of the SDP *Pharbitis nil* (14). The steady-state amount of *PnGLP* transcripts increases transiently during flower-inductive darkness and peaks at a time that corresponds approximately to the critical night length. When the flowering response is lowered by a night-break treatment, the peak is lower but is still present. Interestingly, Ono *et al.* (14) compared *SaGLP* and *PnGLP* : in *Sinapis*, the expression of *SaGLP* peaks 12h after light is turned on - i.e. during the light period in LD - and is damped in darkness, while in *Pharbitis*, the expression of *PnGLP* peaks 10 h after light is turned off - i.e. during the dark period in SD - and is damped in light. Thus, the pattern of *GLP* expression correlates with the photoperiodic response type. The function of these proteins is still undetermined. Germin synthesis is found to be induced at the onset of growth in germinating wheat embryos and has recently been shown to possess oxalate oxidase activity, releasing H_2O_2 and Ca^{2+} from Ca^{2+} oxalate (see 6).

In tobacco, a polypeptide doublet of *ca* 22 kDa was shown to accumulate in leaf plasma membranes around the floral induction period, under continuous 16-h LD (5). Although no significant homologies were found in databases for the corresponding cDNAs and despite the fact that flowering was not strictly controlled, one may wonder whether these convergent observations on different plant species are simply mere coincidences.

*Sa*GRPs have an RNA-Recognition Motif (RRM) and their transcripts show alternative splicing (7). They were located in nuclei, thus have a putative regulatory role. *In situ* hybridisation with antisense mRNA showed expression in young and meristematic tissues (including the shoot apical meristem and leaf primordia). Recently, the *FCA* gene was cloned from a late-flowering mutant of *Arabidopsis* (12). The FCA product was identified as an RNA-binding protein (with 2 RRMs) and its transcript was also found to undergo alternative processing. These observations suggest that post-transcriptional regulation could be an important mechanism in the control of flowering.

The *Arabidopsis* counterpart of *SaGRP1* corresponds to *AtGRP7*, independently and serendipitously cloned by 2 other groups (one group called it *CCR2)*. In *Arabidopsis*, the steady-state level of *AtGRP7* transcripts oscillates in light/dark cycles and in LL, with the same kinetic than *SaGRP1* in *Sinapis* (8). On the other hand, the concentration of the *At*GRP7 protein oscillates - with a 4-h delay in comparison with its transcripts - while *Sa*GRP1 does not. Stabilising the amount of protein at a high level by over-expressing *AtGRP7* causes the circadian oscillation of the endogenous transcripts to faint, suggesting that *At*GRP7 may exert a negative feedback onto the transcription of its own gene. This is a property generally assigned to internal oscillators. However, since other circadian-regulated genes - *CAB* genes - still cycle in these transgenics, *AtGRP7* is thought to be a 'slave' rather than a 'central' oscillator.

The real implication of these genes in floral induction has yet to be demonstrated. For example, the spatial patterns of *SaGRP*s and *SaGLP* do not fulfil our expectations : they are much less abundant in mature than in young leaves although mature leaves are known as the main sites

of photoperiod perception. Hence we are looking forward to know where the homologues are expressed in *Arabidopsis* and what is the floral behaviour of the *AtGRP7* overexpressing plants. Constitutive expression of another gene of *Arabidopsis* recently proposed to be a clock-component (*LHY*) gives a late-flowering phenotype under LD only (3).

As mentioned above, *AtGRP7* (or *CCR2)* had already been cloned by 2 groups whose interest was not flowering at all. *AtGRP7* was isolated from a cDNA library screened with a wheat ubiquitin conjugating enzyme (18) and *CCR2* was cloned from leaves inoculated with turnip crinkle virus although it was shown not to be induced by the pathogen (1). People working on *CCR2* had observed its circadian-regulation and asked the question whether this gene was regulated by the same time keeper than *CAB* genes (10). Their purpose was to determine if rhythms which are out of phase are mediated by either 2 clocks or by a single clock with different output pathways. They therefore compared the effects of a transient cold treatment on the oscillation of both transcript families and concluded that *CCR* and *CAB* genes share clock machinery.

What a coincidence again ! Leaving floral induction in *Sinapis* to arrive to *CAB* gene expression, with *AtGRP7* in between and the question : 'Are they controlled by the same clock ?' I was really amazed ! We indeed addressed the same question in our lab: 'Are flowering and *CAB* gene expression under the control of the same clock ? '. The material on which we investigated this problem was the qualitative LDP *Lolium temulentum* Ceres where the photoperiodic control of flowering was shown to imply a rhythmic sensitivity to light (15) and where many transcripts potentially related to the biological clock were found to be affected by the inductive LD (17). The strategy we used was (coincidence again ?) to expose plants of *Lolium* to an 8-h cold treatment (2-4°C) before assessing the flowering response to a light break and the circadian expression of *CAB* genes (16). Our results also showed common effects on both rhythms : cold delayed what should have been happening during its application but did not disturb the timings themselves. Thus, clock(s) driving photoperiodic induction of flowering and *CAB* gene expression share in common an insensitivity to cold in *Lolium*.

Beside our physiological approach, other researchers use genetics to determine whether the circadian clock, that times well characterised circadian rhythms, is a component of the daylength sensing system. One elegant screening of clock mutants uses as a marker a fusion between the circadian-regulated promoter of the *Arabidopsis CAB2* gene and a firefly luciferase reporter gene (2). The results obtained so far with these *toc* (*timing of CAB*) mutants suggest that the same pacemaker mediates *CAB* gene expression and the measurement of photoperiod (2).

Altogether, the investigations described above connect the photoperiodic induction of flowering with the biological clock at the molecular level. In some of them, the connection - that had been previously demonstrated at the physiological level - was used as a handle to unravel the mechanisms of floral induction. However, when the process was investigated the other way round - looking for transcripts whose abundance changes with photoperiod - many circadian-regulated transcripts were also picked up. This was shown by cDNA cloning and differential screening in the SDP *Pharbitis* (13,19). Three clones were selected for further investigation and shown to exhibit circadian-regulation. One of them encodes a high mobility group DNA-binding protein (19), the other 2 - PN1 and PN9 - have unknown functions (13). In the LDPs *Arabidopsis* (11), *Sinapis* (4), and *Lolium* (17), 2D-PAGE analysis of *in vitro* translated products was used to follow mRNA patterns in SD and in LD. In all 3 cases, lengthening of the light period that induces flowering led to a transient modification of many rhythms observed in SD. Only in *Arabidopsis* were fluctuations observed in DD, allowing Lechner and Rau to assert that the transcripts under study were circadian-regulated (11). In the studies on *Sinapis* and *Lolium*, a relation between the transcripts responsive to the LD and the biological clock was assumed because their fluctuations in SD did not follow environmental cues (4,17).

Now, I am leaving you to add your own cross-talks ... Maybe you will be convinced -

as I am - that people investigating gene expression during photoperiodic induction of flowering will inevitably be directed to the biological clock, irrespective of the experimental strategy they follow. Chronobiology could thus be as fashionable as molecular genetics !

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