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REVIEW: PART OF A SPECIAL ISSUE ON FLOWER DEVELOPMENT

RAV genes: regulation of floral induction and beyond

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• **Background** Transcription factors of the RAV (RELATED TO ABI3 AND VP1) family are plant-specific and possess two DNA-binding domains. In *Arabidopsis thaliana*, the family comprises six members, including TEMPRANILLO 1 (TEM1) and TEM2. Arabidopsis RAV1 and TEM1 have been shown to bind bipartite DNA sequences, with the consensus motif C(A/C/G)ACA$(N)_{2-8}$(C/A/T)ACCTG. Through direct binding to DNA, RAV proteins act as transcriptional repressors, probably in complexes with other co-repressors.
• **Scope and Conclusions** In this review, a summary is given of current knowledge of the regulation and function of *RAV* genes in diverse plant species, paying particular attention to their roles in the control of flowering in arabidopsis. TEM1 and TEM2 delay flowering by repressing the production of two florigenic molecules, FLOWERING LOCUS T (FT) and gibberellins. In this way, TEM1 and TEM2 prevent precocious flowering and postpone floral induction until the plant has accumulated enough reserves or has reached a growth stage that ensures survival of the progeny. Recent results indicate that TEM1 and TEM2 are regulated by genes acting in several flowering pathways, suggesting that TEMs may integrate information from diverse pathways. However, flowering is not the only process controlled by RAV proteins. Family members are involved in other aspects of plant development, such as bud outgrowth in trees and leaf senescence, and possibly in general growth regulation. In addition, they respond to pathogen infections and abiotic stresses, including cold, dehydration, high salinity and osmotic stress.

Key words: RAV family, *TEMPRANILLO* genes, flowering, arabidopsis development, transcription factors, biotic/abiotic stress, photoperiod, gibberellins, flower development.

INTRODUCTION

Flowering must occur at an appropriate time of the year to ensure offspring survival and species perpetuation. A delay in floral induction may lead to a robust plant, but be late for seed maturation. By contrast, a precocious flowering will result in a plant without enough energy for the development of fruits. Therefore, the time for floral induction is critical, and consequently both late induction and precocious flowering should be avoided. Plants respond to seasonal changes in daylength and temperature. In both inductive and non-inductive conditions flowering must be postponed until the plant obtains enough reserves for flower formation, and in unfavourable conditions it must be delayed to reach the appropriate time for seed-set. *Arabidopsis thaliana* is a good model to study this process. It is a facultative long day (LD) plant, i.e. it flowers rapidly when days are long, such as in spring, but it also eventually flowers in short days (SD). Several genetic pathways control flowering time in response to environmental or endogenous conditions. The major environmental effectors are daylength or photoperiod, seasonal and daily changes in temperature, and light intensity and quality (Thomas, 2006; Andrés and Coupland, 2012; Song *et al*., 2012, 2013). Among the endogenous factors are hormones such as gibberellins (GAs) and the age of the plant (Mutasa-Göttgens and Hedden, 2009; Huijser and Schmid, 2011). These pathways have been studied extensively in arabidopsis. The information provided by these genetic pathways is integrated in the activation of the expression of the so-called floral pathway integrators, *FLOWERING LOCUS* T (*FT*) and *SUPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*), which trigger flowering (Fornara *et al*., 2010; Wellmer and Riechmann, 2010). A major inducer of flowering in response to long days is CONSTANS (CO). *CO* transcript levels are high at the end of the light period under LD and its protein is stabilized only under light. If the expression coincides with the dark period, as in SD, the protein is immediately degraded. Therefore, CO is only active under LD (Suárez-López *et al*., 2001; Valverde *et al*., 2004; Jang *et al*., 2008; Liu *et al*., 2008).

Leaves perceive light and other environmental conditions, and CO is expressed in their vascular tissue, where it activates *FT* transcription (Takada and Goto, 2003; An *et al*., 2004). FT protein, identified as part of the florigen, travels to the shoot apical meristem (SAM), where flowers will be produced, to induce flowering (Corbesier *et al*., 2007; Jaeger and Wigge, 2007; Lin *et al*., 2007; Mathieu *et al*., 2007; Tamaki *et al*., 2007). In addition to FT, GAs are also mobile signals that travel from the leaves to the SAM to induce *FT* and *SOC1* in order to trigger flowering (Eriksson *et al*., 2006). Different enzymatic activities give rise to the bioactive GA form GA_4 (Mutasa-Göttgens and Hedden, 2009). As mentioned, these mobile inductive signals should be repressed for the correct timing of flowering. Several proteins have been identified as repressors and together prevent precocious flowering (Jarillo and Piñeiro, 2011). Two of these proteins are

TEMPRANILLO1 (TEM1) and TEM2 (Castillejo and Pelaz, 2008; Osnato *et al*., 2012), which belong to the RAV (Related to ABI3/VP1) family of transcription factors.

Here we review the role of *RAV* genes in different species and show that they are involved in several plant processes such as flowering, bud outgrowth, leaf senescence, responses to hormones, stress and other environmental signals.

RAV FAMILY OF TRANSCRIPTION FACTORS

In arabidopsis there are six members of the RAV family of transcription factors: RAV1, RAV1-like, RAV2, RAV2-like, RAV3 and RAV3-like (Fig. 1A) (Riechmann *et al*., 2000). The first four have also been named ETHYLENE RESPONSE DNA BINDING FACTORS (EDF1–EDF4) (Alonso *et al*., 2003). Based on their function in flowering, *RAV2-like* and *RAV2* were renamed *TEM1* and *TEM2*, respectively (Castillejo and Pelaz, 2008). The main characteristic of RAV members is the presence of two different DNA-binding domains, a B3 and an AP2 domain (Fig. 1B). RAV family members have thus been classified as members of either the B3 super-family or the AP2/EREBP (APETALA2) family of transcription factors.

The B3 domain was initially identified in the VIVIPAROUS1 (VP1) protein from *Zea mays*, and in the ABSCISIC ACID INSENSITIVE3 (ABI3), the VP1 orthologue from arabidopsis (Giraudat *et al*., 1992; Suzuki *et al*., 1997). B3 domains, consisting of a seven-stranded β-sheet arranged in an open barrel and two short α helices, generally share a common structural framework for DNA recognition (Yamasaki *et al*., 2004; Waltner *et al*., 2005). As mentioned, the RAV proteins are characterized by the presence of not only a C-terminal B3 domain that recognizes the consensus CACCTG sequence, but also an N-terminal AP2 domain that recognizes the consensus CAACA sequence (Kagaya *et al*., 1999). The AP2 domain is about 60 amino acids (aa) long (Okamuro *et al*., 1997; Riechmann and Meyerowitz, 1998; Riechmann *et al*., 2000; Sakuma *et al*., 2002; Magnani *et al*., 2004). This makes the RAV transcription factors unique, with two different DNA binding domains (Fig. 1B).

The contribution of transcriptional repressors may be of crucial importance in various plant biological processes. Around 10 % of arabidopsis transcription factors might be transcriptional repressors (Ikeda and Ohme-Takagi, 2009). Among the B3 super-family, it was found that many members had a repressive activity due to the existence of a 15-aa peptide (GNSKTLRLFGVNMEC), which has been named the B3 repression domain (BRD). Although replacement experiments pointed to the first leucine and/or the methionine residue (in bold) of the BRD (GNSKT**L**RLFGVN**M**EC) as crucial to maintain repressive activity, other amino acids of this domain are not always conserved. Deletion of the BRD of some B3 proteins revealed that only a short peptide of five amino acids, R/KLFGV, is essential as a repression domain. Four members of the RAV family, TEM1, TEM2, RAV1 and RAV1-like, share the core of the BRD (Ikeda and Ohme-Takagi, 2009). A quite similar sequence, MLFGV, is present in RAV3 and RAV3-like (Causier *et al*., 2012). The R/KLFGV sequence is also conserved in other RAV homologues from various plants such as rice (Ikeda and Ohme-Takagi, 2009). These results suggest strongly that RAV genes encoding RLFGV motifs could play roles as transcriptional repressors (Fig. 1B).

TEM GENES REPRESS FLOWERING IN TWO DIFFERENT PATHWAYS

As mentioned, *FT* plays a central role during the floral induction event (Turck *et al*., 2008) and is activated in response to CO (Kardailsky *et al*., 1999; Kobayashi *et al*., 1999; Samach *et al*., 2000). However, CO is already expressed in the phloem early in development (Takada and Goto, 2003), and changes in *CO* expression levels do not seem to account for the increase in *FT* accumulation for inducing flowering (Castillejo and Pelaz, 2008). Consequently, something else that accounts for this late FT accumulation must exist.

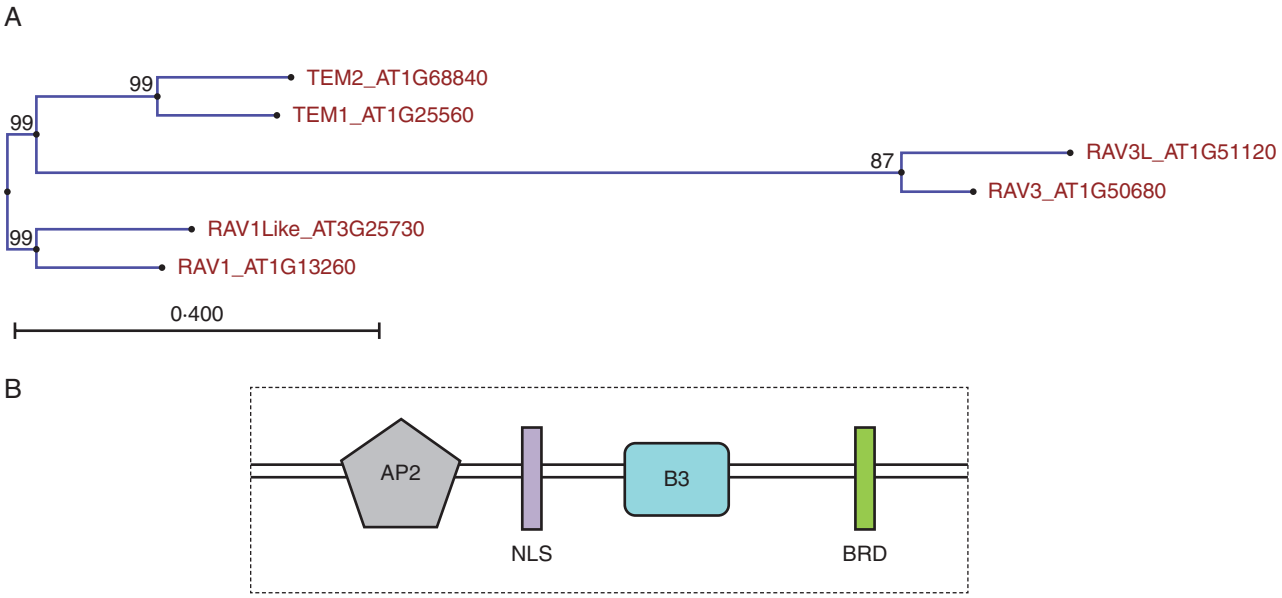
TEM *genes affect the photoperiod pathway*

Regulation of flowering initiation in response to photoperiod is mediated by the interaction between external light signals and the circadian clock (Suárez-López *et al*., 2001; Yanovsky and Kay, 2002). In the photoperiod pathway, FT promotes flowering in response to LD. *TEM1* and *TEM2* were identified as repressors of flowering in the photoperiod pathway (Castillejo and Pelaz, 2008). Single loss-of-function alleles of *TEM1*, *tem1–1*, and *TEM2*, *tem2–2*, cause a slight early flowering phenotype in LD, and a double *tem1–1 tem2–2* mutant shows enhanced early flowering compared with the single mutants under LD conditions. In this photoperiod, *tem1–1 tem2–2* flowers as early as CO overexpressors (*35S::CO*). Supporting these results, it was found that both *35S::TEM1* and 35S::*TEM2* plants show the opposite phenotype and flower extremely late under LD conditions (Castillejo and Pelaz, 2008; Osnato *et al*., 2012). Consequently, TEMs seem to play a pivotal role as repressors in floral induction (Fig. 2).

TEM1 transcript levels follow a diurnal oscillation, such that *TEM1* abundance is low during the daytime and peaks at dusk. Similar developmental and circadian regulations were observed for *TEM1* and *TEM2*, supporting the proposed redundant role of both genes (Castillejo and Pelaz, 2008; Osnato *et al*., 2012). Moreover, *TEM1* mRNA abundance is very high during early stages of seedling development but a pronounced decline takes place just before floral transition. *CO* expression remains almost unaltered throughout development, although a subtle increase occurs during the transition to flowering (Castillejo and Pelaz, 2008).

In addition in wild-type plants, *FT* mRNA remains at basal levels until the transition to flowering, at days 10–12, when there is a pronounced increase in *FT* accumulation. However, *FT* expression increases from day 6 in the *tem1–1 tem2–2* double mutant, when plants had only formed the first two true leaves (Osnato *et al*., 2012). The significant increase of FT expression responsible for floral induction is abolished in the *35S::TEM1* seedlings (Castillejo and Pelaz, 2008). Therefore, TEM1 represses *FT* expression at early developmental stages.

The identical precocious flowering phenotypes of *35S::CO* and *tem1 tem2* plants suggested strongly that only when *TEM* levels drop drastically can CO activate *FT* to reach the threshold level necessary to trigger the floral transition under inductive photoperiods (Castillejo and Pelaz, 2008). When both *CO* and *TEM* levels are elevated, in 35S::*CO 35S::TEM1* plants, the balance between the activator and the repressor is restored and consequently these plants flower after producing a wild-type

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TEM1_AT1G25560      ---MEY-SCVDD-SSTTSESLSISTTPKPTTTTEKKLSSPPATSMR--LYRMGSGGSSVV
TEM2_AT1G68840      ---MDS-SCIDEISSSTSESFS--------ATTAKKLSPPPAAALR--LYRMGSGGSSVV
RAV1_AT1G13260      ---MES-SSVDE-STTSTGSIC-----------ETPAITPAKKSSVGNLYRMGSG-SSVV
RAV1Like_AT3G25730  ---MDAMSSVDE-SSTTTDSIP-----------ARKSSSPAS-----LLYRMGSG-TSVV

TEM1_AT1G25560      LDSENG----VETESRKLPSSKYKGVVPQPNGRWGAQIYEKHQRVWLGTFNEEEEAASSY
TEM2_AT1G68840      LDSENG----LETESRKLPSSKYKGVVPQPNGRWGAQIYEKHQRVWLGTFNEQEEAARSY
RAV1_AT1G13260      LDSENG----VEAESRKLPSSKYKGVVPQPNGRWGAQIYEKHQRVWLGTFNEEDEAARAY
RAV1Like_AT3G25730  LDSENGVEVEVEAESRKLPSSRFKGVVPQPNGRWGAQIYEKHQRVWLGTFNEEDEAARAY

TEM1_AT1G25560      DIAVRRFRGRDAVTNFKSQVDGN-DAESAFLDAHSKAEIVDMLRKHTYADEFEQSRRK--
TEM2_AT1G68840      DIAACRFRGRDAVVNFKNVLE---DGDLAFLEAHSKAEIVDMLRKHTYADELEQNNKRQL
RAV1_AT1G13260      DVAVHRFRRRDAVTNFKDVKMD--EDEVDFLNSHSKSEIVDMLRKHTYNEELEQSKRR--
RAV1Like_AT3G25730  DVAAHRFRGRDAVTNFKDTTF---EEEVEFLNAHSKSEIVDMLRKHTYKEELDQRKRN--

TEM1_AT1G25560      --FVNGDGKRSGLETATYG-----NDAVLRAREVLFEKTVTPSDVGKLNRLVIPKQHAEK
TEM2_AT1G68840      FLSVDANGKRNG--SSTTQ-----NDKVLKTREVLFEKAVTPSDVGKLNRLVIPKQHAEK
RAV1_AT1G13260      ---RNGNGNMTRTLLTSGLSNDGVSTTGFRSAEALFEKAVTPSDVGKLNRLVIPKHHAEK
RAV1Like_AT3G25730  ---RDGNGKETTAFALASM----VVMTGFKTAELLFEKTVTPSDVGKLNRLVIPKHQAEK

TEM1_AT1G25560      HFPLPAMTTA---MGMNPSPTKGVLINLEDRTGKVWRFRYSYWNSSQSYVLTKGWSRFVK
TEM2_AT1G68840      HFPLPSPS---------PAVTKGVLINFEDVNGKVWRFRYSYWNSSQSYVLTKGWSRFVK
RAV1_AT1G13260      HFPLPS----------SNVSVKGVLLNFEDVNGKVWRFRYSYWNSSQSYVLTKGWSRFVK
RAV1Like_AT3G25730  HFPLPLGN--------NNVSVKGMLLNFEDVNGKVWRFRYSYWNSSQSYVLTKGWSRFVK

TEM1_AT1G25560      EKNLRAGDVVCFERSTGPD--------RQLYIHWKVRS----SPVQTVVRLFGVNIFNVS
TEM2_AT1G68840      EKNLRAGDVVCFERSTGLE--------RQLYIDWKVRSGPRENPVQVVVRLFGVDIFNVT
RAV1_AT1G13260      EKNLRAGDVVCFERSNGQD--------QQLYIGWKSRSGS-DLDAGRVLRLFGVNISPES
RAV1Like_AT3G25730  EKNLCAGDLISFKRSNDQD--------QKFFIGWKSKSGL-DLETGRVMRLFGVDIS---

TEM1_AT1G25560      NEKPNDVAVECVGKKRSREDDLFSLGCSKKQAIINIL
TEM2_AT1G68840      TVKPNDVVAVCGGKRSRDVDDMFALRCSKKQAIINAL
RAV1_AT1G13260      S--RNDVVGN----KRVNDTEMLSLVCSKKQRIFHAS
RAV1Like_AT3G25730  ---LNAVVVV----KETTEVLMSSLRC-KKQRVL---
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FIG. 1. (A) Phylogenetic unrooted tree of the RAV family in *Arabidopsis thaliana.* Analysis was done using the CLC Genomics Workbench v6.5 program; bootstrap values are indicated. (B) Amino acid sequence of four RAV members, TEM1, TEM2, RAV1 and RAV1L, with the exact location of these domains. Analysis was done using Clustal v2.1 multiple amino acid alignment; substitution rate model = WAG. Main characteristic protein domains of RAV transcription factor family include: *AP2* (grey) and *B3* (blue) DNA-binding domains; nuclear localization signal (*NLS*) in purple; and B3 repression domain (*BRD*) in green.

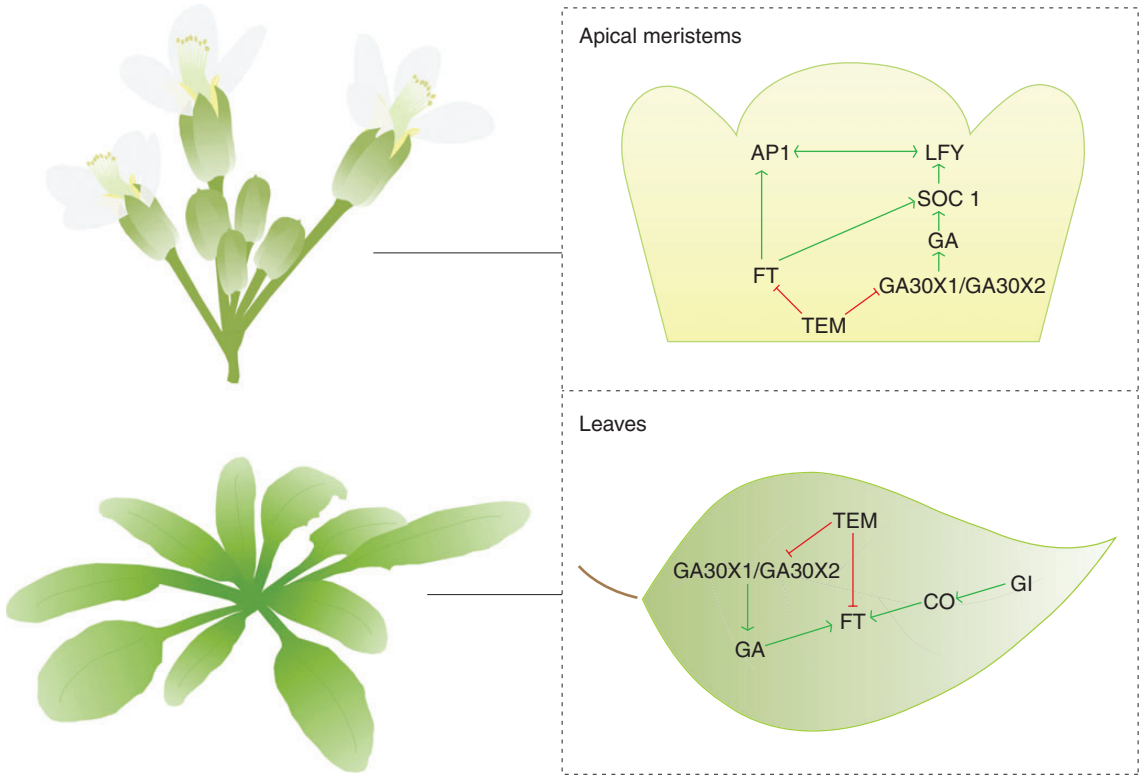

FIG. 2. Floral transition model in *Arabidopsis thaliana*. *TEM1* and *TEM2* genes play a central role in regulating the flowering process by repressing at least the photoperiod and gibberellin pathways under inductive and non-inductive daylengths, in leaves and apical meristems.

number of leaves. The late-flowering phenotype of *35S::*TEM1 plants is completely suppressed by the constitutive expression of *FT*, which is consistent with FT acting downstream of *TEM1*. The combination of *tem1–1* and *ft* mutants confirmed the epistatic relationship between both genes, as the double mutant *tem1–1 ft-101* flowers at the same time as *ft-101* alone (Castillejo and Pelaz, 2008). These results also suggest that *FT* is the primary downstream target of TEM1 to repress flowering.

TEM1 expression is detected in all vegetative tissues (Castillejo and Pelaz, 2008). It has been proposed that TEM could act in the vascular bundles of leaves, together with CO, to tightly control FT accumulation; however, *TEM1* is expressed throughout the leaf as well as in the SAM and the hypocotyl. An artificial micro RNA (amiRNA) targeted against *TEM1* and *TEM2* genes was expressed under control of the *KNAT1* promoter to drive their silencing only in the SAM and hypocotyls. An early flowering phenotype of *pKNAT1::amiR-TEM* lines was associated with an up-regulation of *FT* expression. All this indicated that TEM has a role in controlling flowering, at least in the SAM (Osnato *et al*., 2012).

RAV binding motifs (Kagaya *et al*., 1999) were found in the 5′ untranslated region (UTR) of the *FT* gene. *In vitro* and *in vivo* interactions of TEM1 protein with the *FT* 5′UTR were confirmed by gel-shift and chromatin immunoprecipitation (ChIP) assays, respectively. Interestingly, the RAV binding site in *FT* is located just next to the CO binding site found 43 bp upstream of the ATG (Wenkel *et al*., 2006; Castillejo and Pelaz, 2008). Therefore, precise control of flowering time could be explained if the CO and TEM proteins compete for their respective binding sites to directly regulate *FT* accumulation. Consequently, *FT* levels are the result of a quantitative balance between the respective promoter and repressive activities of CO and TEM (Castillejo and Pelaz, 2008).

GIGANTEA (GI), a circadian clock regulator, plays a role in floral induction through regulation of the timing and amplitude of *CO* expression (Fowler *et al*., 1999; Park *et al*., 1999; Mizoguchi *et al*., 2005; Sawa *et al*., 2007). GI and FLAVIN-BINDING, KELCH REPEAT, F BOX protein 1 (FKF1) form a protein complex that mediates the degradation of CYCLING DOF FACTOR 1 (CDF1), a key *CO* repressor. Under LDs, GI and FKF1 expression peak at the same time, at the end of the day, leading to the optimal formation of the GI–FKF1 complex. However, under SDs, the expression of GI peaks a few hours before the peak of FKF1 expression, resulting in low levels of the GI–FKF1 complex and maintenance of the repressor CDF1 (Sawa and Kay, 2011).

CO and *FT* are mainly expressed in vascular tissue, whereas (and similarly to *TEM* genes) *GI* is expressed in various tissues including vascular bundles, mesophyll, SAM and root (Takada and Goto, 2003; An *et al*., 2004; Winter *et al*., 2007). In fact, *GI* expression in either mesophyll and/or vascular tissue rescues the late-flowering phenotype of the gi-2 mutant under both SD and LD conditions (Sawa and Kay, 2011). It was observed that the GI N-terminal region was able to interact with TEM1 and TEM2 through yeast-two-hybrid (Y2H) assays. Moreover, the *in vivo* physical interactions of these proteins were found to take place in the nucleus but not in the cytosol (Sawa and Kay, 2011). These authors also showed that

GI activates *FT* expression independently of CO through direct binding to *FT* promoter regions (alone or in a complex with another protein). A possible explanation is that GI could neutralize the TEM1 and TEM2 repressors by interfering with their access to the *FT* promoter or their activity and/or stability.

TEM *genes also regulate the GA pathway*

By contrast, under SD conditions, in which CO is inactive, flowering is induced in the SAM by GAs through activation of the floral integrator *SOC1*, and the floral meristem identity gene *LEAFY* (*LFY*) (Blazquez and Weigel, 2000; Moon *et al*., 2003; Mutasa-Göttgens and Hedden, 2009). Under SDs, *tem1–1 tem2–2* double mutants still flower much earlier than wild-type plants. When expression levels of *SOC1* and *LFY* are analysed in wild-type and *tem1–1 tem2–2* mutant plants under SDs, a significant enhancement of *SOC1* and *LFY* expression is observed in *tem1–1 tem2–2*, indicating an additional role of TEM in flowering-time regulation under SD conditions (Osnato *et al*., 2012). By contrast, *35S::TEM1* plants flower extremely late under SDs, most of them remaining at the vegetative phase and producing leaves indefinitely. In this photoperiod, *TEM* mRNA levels are low during the light period, start to increase at dusk and peak early in the night in wild-type plants. *TEM1* and *TEM2* expression patterns are similar, except for an extra *TEM2* peak late at night (Osnato *et al*., 2012).

pKNAT1::amiR-TEM plants have elongated hypocotyls both in LD and in SD conditions, while *35S::TEM1* plants show, apart from the extremely late flowering, a dwarf phenotype, loss of apical dominance and shorter hypocotyls (Osnato *et al*., 2012). These are phenotypes typical of GA-deficient mutants, such as *ga3ox1–3* and the double mutant *ga3ox1 ga3ox2* (Eriksson *et al*., 2006; Mitchum *et al*., 2006). When GA is sprayed onto the *35S::TEM1* plants the apical dominance and flowering phenotypes are rescued (Osnato *et al*., 2012), suggesting that *TEM* genes play a major role in the GA pathway.

Furthermore, a significant down-regulation of *GA20OX2*, *GA3OX1* and *GA3OX2* expression is found in *35S::TEM1*, whereas an up-regulation of *GA3OX1* and *GA3OX2* is observed in *tem1–1* and *tem1–1 tem2–2* in comparison with the wild type (Osnato *et al*., 2012). *35S::TEM1* produces a down-regulation of GUS expression in plants carrying a *GA3OX1::*GUS reporter construct (Mitchum *et al*., 2006), specifically in the SAM of young plants and in leaves of older plants (Osnato *et al*., 2012). These results indicate a clear effect of TEM on the enzymes that catalyse the last step of GA_4 biosynthesis. In addition, ChIP assays show that TEM1 is a direct *in vivo* regulator of the GA_4 biosynthetic genes *GA3OX1* and *GA3OX2* by binding an RAV binding site positioned in the first exon in both cases (Osnato *et al*., 2012). These data therefore corroborate that TEM directly represses *GA3OX* genes, which may result in a reduction of bioactive GA_4. *tem1 tem2 ga3ox1* triple mutant plants flower later than *tem1 tem2* plants but still earlier than the wild type and *ga3ox1* single mutant, indicating that the early flowering phenotype of *tem1 tem2* double mutants in LD is due at least partially to the *GA3OX1* up-regulation (Osnato *et al*., 2012), which also indicates that GAs act both in LD and in SD.

In conclusion, *TEM* genes link the photoperiod- and GA-dependent flowering pathways, controlling the floral transition under inductive and non-inductive daylengths (Fig. 2).

OTHER RAV FAMILY MEMBERS MAY AFFECT FLOWERING

Results with RAV1 antisense lines suggest that RAV1 may be a flowering repressor in arabidopsis (Hu *et al*., 2004). However, it has not been shown whether the full-length antisense construct used to generate these antisense lines is specific for RAV1. Levels of other *RAV* family transcripts should be checked in these plants to discard the possibility that the early flowering is due to off-target effects on *TEM1* and/or *TEM2*. It is also possible that RAV1 antisense plants flower a few days earlier than wild-type plants as a result of differences in the rate of leaf production (Hu *et al*., 2004).

When GmRAV, a soybean (*Glycine max*) TEM/RAV homologue, is overexpressed in tobacco (*Nicotiana tabacum*) it delays flowering. This suggests that GmRAV, similar to TEM1 and TEM2, can act as a flowering repressor. Although soybean flowering is promoted by SD, *GmRAV* shows higher expression under SD than under LD (Zhao *et al*., 2008). They proposed that the repression of flowering by GmRAV in tobacco may indirectly result from negative effects on photosynthesis and other aspects of plant physiology. Further research should determine whether GmRAV is a regulator of flowering.

RAV GENES ARE REGULATED BY DIFFERENT FLOWERING PATHWAYS

Age-dependent flowering pathway

Genes involved in several flowering pathways regulate *TEM*/*RAV* genes. Several *AP2* family genes are targets of the miRNA miR172 and encode floral repressors that act in the photoperiod- and the age-dependent flowering pathways. In arabidopsis these repressors include AP2 itself, TARGET OF EAT1 (TOE1), TOE2, TOE3, SCHLAFMÜTZE (SMZ) and SCHNARCHZAPFEN (SNZ) (Zhu and Helliwell, 2011). AP2 and SMZ bind *TEM1* chromatin in ChIP-chip experiments (Mathieu *et al*., 2009; Yant *et al*., 2010), suggesting that they may induce *TEM1* expression. However, *TEM1* mRNA levels are not altered in the leaves and the shoot meristem of an activation-tagged *smz-D* mutant, which flowers later than the wild type (Mathieu *et al*., 2009). By contrast, *TEM2* is upregulated in *smz-D*, despite not being bound by SMZ in ChIP-chip experiments (Mathieu *et al*., 2009). These observations suggest that TEM1 and TEM2 may mediate at least part of the effects of AP2 and SMZ on flowering, although additional experiments are required to demonstrate this.

TOPLESS (TPL) and TPL-related (TPR) proteins constitute a family of five members that interact with diverse transcription factors and act as transcriptional co-repressors in arabidopsis (Long *et al*., 2006; Szemenyei *et al*., 2008). TOE1, TOE2 and AP2 are among these TPL/TPR-interacting transcription factors (Arabidopsis Interactome Mapping Consortium, 2011; Causier *et al*., 2012; Krogan *et al*., 2012). Overexpression of TOE1 delays flowering and TPL is required for this phenotype, suggesting that TPL, and perhaps also TPRs, acts as a co-repressor of flowering (Causier *et al*., 2012). Interestingly, all members of the RAV family, with the exception of RAV1L, also interact with TPL/TPR proteins. The RLFGV or MLFGV domains present in all RAV proteins (see above) are required for the interaction of at least RAV1 and RAV3L with TPL (Causier *et al*., 2012). Therefore, RAV proteins probably act in complexes

with TPL/TPR to repress transcription of floral regulators. The action of TPL and its homologues in mammals and yeast involves histone deacetylation and chromatin condensation (Long *et al.*, 2006; Krogan *et al.*, 2012; Turki-Judeh and Courey, 2012; Wang *et al.*, 2013). It will be interesting to determine whether the mechanism of transcriptional repression by RAV proteins also implies chromatin remodelling through the recruitment of TPL/TPR.

Ambient temperature pathway

Changes in ambient temperature affect flowering and low temperatures delay the floral transition in arabidopsis (Blazquez *et al.*, 2003). EARLY FLOWERING 3 (ELF3) is a repressor of flowering involved in this response (Strasser *et al.*, 2009). *elf3* mutants flower earlier and are less sensitive to temperature than wild-type plants, such that the delay caused by low temperature is smaller in *elf3* than in the wild type. *TEM2* is downregulated in *elf3* both at 16 and at 23 °C (Strasser *et al.*, 2009), which correlates with the early flowering phenotype at both temperatures. In addition, the downregulation of *TEM2* in *elf3* is more dramatic at 16 than at 23 °C (Strasser *et al.*, 2009), consistent with a bigger difference in flowering time between *elf3* and the wild type at 16 than at 23 °C. This suggests that the repression of flowering by ELF3 may be mediated at least in part by an increase in *TEM2* expression. *RAV1* shows lower transcript levels in *elf3* than in the wild type at 16 °C, but higher expression at 23 °C, indicating that *RAV1* expression is also regulated by ELF3.

Two MADS-box transcription factors, FLOWERING LOCUS C (FLC) and SHORT VEGETATIVE PHASE (SVP), form a complex that represses flowering during vegetative growth (Li *et al.*, 2008). FLC and SVP have both overlapping and distinct functions (Balasubramanian *et al.*, 2006; Lee *et al.*, 2007*b*; Li *et al.*, 2008). Both are involved in responses to ambient temperature. SVP is important for the repression of flowering at low ambient temperature, while FLC suppresses the induction of flowering by high temperatures (Balasubramanian *et al.*, 2006; Lee *et al.*, 2007*b*). FLC also plays an important role in vernalization, a response to long periods of cold that induces flowering after winter has passed (Song *et al.*, 2012). In addition, FLC acts in the autonomous flowering pathway (Simpson, 2004). ChIP-seq experiments revealed that FLC binds to the promoter of *TEM1*, although *TEM1* mRNA levels were not altered in an *flc* mutant (Deng *et al.*, 2011). *TEM1* and *TEM2* chromatin is also bound by SVP, which up-regulates expression of these two genes (Tao *et al.*, 2012). Therefore, the FLC–SVP complex may positively regulate at least *TEM1* through direct binding to the *TEM1* promoter. It would be interesting to test whether TEM1 and/or TEM2 affect the response of flowering to ambient temperature and/or vernalization. Although SVP and FLC had initially been described as transcriptional repressors (Hepworth *et al.*, 2002; Gregis *et al.*, 2006), they also seem capable of inducing transcription, including that of other flowering repressors in addition to *TEM1* and *TEM2* (Deng *et al.*, 2011; Tao *et al.*, 2012). The mechanism of this positive regulation remains unknown, but probably contributes to reinforce the repression of flowering under unfavourable conditions.

Another MADS-box protein with an important role in flowering-time control, SOC1, regulates *TEM1* and *TEM2* expression, but in the opposite way to the regulation by SVP. Regulatory regions of the *TEM1* and *RAV1* genes are bound by SOC1, indicating that the effect of SOC1 on at least *TEM1* is probably direct (Tao *et al.*, 2012). The repression of *TEM1* and *TEM2* by SOC1 is consistent with the induction of flowering by SOC1.

Brassinosteroids

Brassinosteroids (BRs) are a class of steroid hormones that regulate many developmental processes throughout plant life, such as vascular development, senescence and flowering. Mutants with altered content in endogenous BRs, such as *deetiolated2* or *dwarf4*, flower late, indicating that components of the BR pathway also affect flowering time (reviewed by Li *et al.*, 2010). Treatment with BR reduces *RAV1* and *GmRAV* transcript levels in arabidopsis and in soybean, respectively (Hu *et al.*, 2004; Zhao *et al.*, 2008), indicating that BR down-regulates these genes. In arabidopsis, the effect of BR on *RAV1* seems independent of the BR receptor BRI1 (Hu *et al.*, 2004), suggesting that other BR receptors may be involved. The effect of BR on flowering might therefore be mediated by RAV family members. Given that BR affects many aspects of plant development and growth, additional research is required to determine in which aspect *TEM*/*RAV* genes may be involved.

Although the rice *SVP* group of genes seems not to be involved in flowering, they do affect BR responses (Duan *et al.*, 2006; Lee *et al.*, 2008). This, together with the regulation of *TEM1* and *TEM2* by SVP, the regulation of *FLC* expression by BR and the binding of FLC to *TEM1* DNA (Domagalska *et al.*, 2007; Deng *et al.*, 2011; Tao *et al.*, 2012), establishes another possible link between BR and *RAV* genes.

Light intensity and quality

In addition to photoperiod, light intensity and quality affect floral induction, as well as many other aspects of plant development and growth (Chen *et al.*, 2004; Thomas, 2006). Several results indicate that *RAV* genes may be involved in light responses.

ELONGATED HYPOCOTYL 5 (HY5) is a transcription factor that promotes photomorphogenesis downstream of several photoreceptors (Oyama *et al.*, 1997). In addition, HY5 represses flowering, as shown by the early flowering of *hy5* mutants (Goto *et al.*, 1991; Holm *et al.*, 2002). *TEM2* expression is positively regulated by HY5, which binds to *TEM1*, *TEM2* and *RAV1* chromatin, suggesting that the regulation of *TEM2* is direct (Lee *et al.*, 2007*a*). Therefore, TEM2 is a good candidate to link HY5 with the regulation of flowering in response to light signals.

EFFECT OF RAV FAMILY MEMBERS ON OTHER ASPECTS OF PLANT DEVELOPMENT

RAV *genes regulate hypocotyl elongation*

Transcriptomic analyses of arabidopsis seedlings grown in continuous white light and in the dark have shown that *TEM2* is up-regulated in the hypocotyl and root in response to light, whereas *RAV1* is down-regulated in cotyledons of light-grown seedlings (Ma *et al.*, 2005). *TEM1*, *RAV1* and *RAV1L* are rapidly repressed upon exposure of dark-grown seedlings to

red light (Monte *et al*., 2004; Leivar *et al*., 2009; Shin *et al*., 2009). Moreover, *TEM2* expression is induced by a short exposure to far-red light (Tepperman *et al*., 2004). These data indicate that *RAV* genes show specificity in their response to different light conditions in different organs.

PHYTOCHROME INTERACTING FACTORS (PIFs) play important roles in the regulation of light responses by the photoreceptors phytochrome A (PHYA) and PHYB (Leivar and Quail, 2011). The repression of *RAV1* and *RAV1L* by red light requires the function of at least PIF3 (Monte *et al*., 2004), and other PIFs are involved in transcriptional regulation of *TEM1* and *TEM2* (Leivar *et al*., 2009). ChIP-seq experiments have identified *TEM2* as a gene bound by PIF5 in plants subjected to low red/far-red light ratio, a condition that simulates shade (Hornitschek *et al*., 2012). Although the relevance of this binding for *TEM2* expression is not yet clear, it suggests that PIF5 might be involved in the regulation of *TEM2* by shade. A quadruple mutant lacking PIF1, PIF3, PIF4 and PIF5 (*pifq*) shows shorter hypocotyls and, under certain conditions, higher *TEM1* and *TEM2* transcript levels than wild-type plants (Leivar *et al*., 2008, 2009). Consistent with this, *tem* mutants and plants overexpressing *TEM1* have longer and shorter hypocotyls than wild-type plants, respectively, under SD (Osnato *et al*., 2012). It remains to be shown whether PIFs affect *TEM2* and/or *TEM1* under this photoperiod, but the fact that PIFs promote hypocotyl growth under SD (Nozue *et al*., 2007) makes this hypothesis plausible. Therefore, TEM1 and TEM2 might play a role in light-regulated growth downstream of PIFs.

RAV *genes might inhibit plant growth*

Overexpression of *TEM1* or *TEM2* in arabidopsis causes dwarfism (Osnato *et al*., 2012). Tobacco plants overexpressing GmRAV (GmRAV-OX) also exhibit smaller leaves and roots and shorter internodes than wild-type plants. Soybean growth is reduced under SD compared with LD, inversely correlated with higher GmRAV levels under SD than LD (Zhao *et al*., 2008). Also, GmRAV causes a reduction in chlorophyll content and photosynthetic rate when overexpressed in tobacco (Zhao *et al*., 2008), which may explain the reduced growth of these plants. These findings suggest that TEM1, TEM2 and GmRAV might repress plant growth. This is consistent with the fact that BR treatment down-regulates GmRAV (Zhao *et al*., 2008). A detailed analysis of plant growth in loss-of-function *tem* mutants and *GmRAV*-silenced lines would be useful to demonstrate whether these genes play a role as growth regulators.

GmRAV might also be involved in root development, as tobacco GmRAV-OX plants develop fewer roots than wild-type plants (Zhao *et al*., 2008). Again, silencing of GmRAV in soybean would help to determine its biological function. Although overexpression of *RAV1* causes a reduction in the number of lateral roots and probably in the rate of leaf production, suggesting that RAV1 may be a negative regulator of plant growth, down-regulation of *RAV1* by an antisense construct does not have a significant effect on these processes (Hu *et al*., 2004).

RAV1 *might regulate leaf senescence*

Leaf senescence, a physiological mechanism affected by many internal and external factors (Lim *et al*., 2007), is strongly regulated by several genes to provide optimal plant fitness. This maximum plant fitness is obtained by remobilizing nutrients from senescent leaves (Woo *et al*., 2010). *In silico* technology has allowed identification of a subset of genes named as the *SENESCENCE-ASSOCIATED GENES* (SAGs). Among these SAGs, *RAV1* was isolated due to the fact that not only *RAV1* but also other *RAV* genes have been associated with leaf maturation and senescence. *RAV1* expression is triggered at a mature stage, reaching maximum expression at an early senescence stage and decreasing at later stages. A similar expression pattern is found for *TEM1*, while for *RAV1L* the expression remains at high level until late senescence (Woo *et al*., 2010). These similar expression patterns during leaf development and senescence suggest a possible redundant role among this family in this aspect. However, neither single loss-of-function mutants of these genes nor the *rav1 tem1* and *rav1 rav1l* double mutants show any significant alteration of the senescence process. By contrast, arabidopsis plants overexpressing *RAV1* under a constitutive promoter show an early age-dependent leaf senescence phenotype as well as one induced by artificial dark (Woo *et al*., 2010). The main senescence-associated physiological markers, such as the degree of leaf yellowing, chlorophyll content and photochemical efficiency, are altered. Moreover, the expression of two senescence marker genes (*SEN4* and *SAG12*) is upregulated in plants overexpressing *RAV1*, whereas *RAV1* expression is induced by senescence-accelerating hormones such as jasmonic acid (JA) and ethylene. Similar results are found in transgenic plants that express *RAV1* under an inducible promoter.

Consequently, these data suggest that at least *RAV1* might play a role during leaf senescence initiation by the activation and/or repression of genes involved in the successful execution of the leaf senescence process (Woo *et al*., 2010). This control could be done by integrating the age-dependent aspects of leaf senescence with senescence-accelerating hormones and environmental influences. Moreover, tobacco *GmRAV*-OX plants show accelerated senescence in response to abscisic acid (ABA) and dark treatments (Zhao *et al*., 2008). Because the analyses of single and double mutants do not demonstrate a role of *RAV* genes in senescence, additional work is required to test whether other family members may control this process in a redundant manner with *RAV1* or *GmRAV*.

The three outer whorls of the flowers in arabidopsis, sepals, petals and stamens, are also organs that senesce and shed after pollination (Chen *et al*., 2011). The time of senescence and organ abscission is controlled by diverse hormones; one of the most important is ethylene, which accelerates this process (Roberts *et al*., 2002). It is known that *FOREVER YOUNG FLOWER* (*FYF*), a MADS transcription factor, acts as a repressor of the ethylene response controlling floral senescence and abscission (Chen *et al*., 2011). Recently, it was found that *TEM1* and *TEM2*, which were previously characterized as downstream genes in the ethylene signalling pathway (Alonso *et al*., 2003), are significantly down-regulated in *35S::FYF* plants. Interestingly, the *FYF* expression pattern is opposite to that of *TEM1* and *TEM2* during flower development. Therefore, these results suggest that FYF controls senescence and organ abscission by inactivating downstream genes in the ethylene response such as *TEM1* and *TEM2* (Chen *et al*., 2011).

RAV *genes control bud outgrowth in trees*

RAV homologous genes have also been identified in trees. A *RAV* gene from chestnut (*Castanea sativa*), *CsRAV1*, has recently been characterized. The closest relatives to *CsRAV1* are two poplar (*Populus thricocarpa*) *RAVs*, *PtRAV1* and *PtRAV2*, and all group with the arabidopsis *TEM1* and *TEM2* genes (Moreno-Cortés *et al*., 2012). Trees are known to have a long juvenile phase when they are still not able to flower. Trees usually form lateral buds that undergo dormancy in the winter period and these buds will grow out the following spring after the cold period. In poplar, sylleptic branching, i.e. outgrowth of branches in the same season in which the buds were formed, is produced and is mainly associated with juvenility (Ceulemans *et al*., 1990; Cooke *et al*., 2005). Tree breeders have long desired to shorten the juvenile phase to speed up breeding and to increase sylleptic branching to obtain a higher woody biomass (Novaes *et al*., 2009; Rae *et al*., 2009). The possibility that *TEM* genes might be involved in the age-dependent pathway in arabidopsis and that this could be conserved across species is of great interest for biomass production in trees. Moreover, the CO/FT module is conserved in *Populus* and, in addition to flowering, regulates bud-set and growth cessation (Böhlenius *et al*., 2006; Hsu *et al*., 2011). This suggests that poplar *TEM* orthologues could be involved in those processes. Although there is still no information on the function of poplar *RAV* genes, the chestnut *CsRAV1* is induced during winter dormancy and in response to low temperatures, which might suggest a role in bud-set and growth cessation; however, more experiments are needed to confirm this. In addition, when *CsRAV1* is overexpressed in hybrid poplar it induces extensive sylleptic branching that it is not observed in control trees (Moreno-Cortés *et al*., 2012). This extra branching greatly increases the biomass of these transgenic trees, which is consequently of agronomic and commercial interest.

RAV GENES AS INTERACTORS OF RESPONSES TO BIOTIC AND ABIOTIC STRESS

Plants, using a complex system, defend themselves against both biotic and abiotic stresses. Plants are able to adapt and survive under several types of biotic and abiotic stresses, such as drought, high salinity, high/low temperatures or pathogen attacks. Worldwide crop productivity and quality are threatened by this wide variety of stresses, and therefore a better understanding of the complex and interconnected systems of plant defence and adaptation to these stresses is crucial. It is known that plants respond to such stresses by inducing morphological, physiological and biochemical changes through crosstalk among different genetic pathways (Zhuang *et al*., 2011). The activation of plant defence responses is first initiated by the recognition/identification of primary pathogen-derived elicitors by plant cell receptors (Yang *et al*., 1997; Kim and Martin, 2004). This triggers signal transduction pathways regulated by the hormones ethylene, salicylic acid (SA) and JA (Glazebrook, 1999; Lee *et al*., 2005), which induce the expression of plant defensive genes that produce defensive compounds, such as pathogen-related (PR) proteins, chitinase and/or enzymes involved in the biosynthesis of protective secondary metabolites (Gu *et al*., 2002; Koo *et al*., 2007).

In recent years, it has been discovered that *RAV* family members from different plant species not only are induced by ethylene but also play essential roles in biotic and abiotic environmental stresses (Alonso *et al*., 2003; Feng *et al*., 2005; Kim *et al*., 2005; Sohn *et al*., 2006; Zhuang *et al*., 2011). For instance, *RAV1* and *TEM2* expression in arabidopsis is upregulated by touch-related stimuli such as touch, wind and water spray, suggesting that these genes may function for developmental adaptation in response to different environmental stimuli (Kagaya and Hattori, 2009). In fact, it was found that expression of both genes is induced in arabidopsis after treatment with biotic and abiotic stresses such as bacterial pathogens, SA, mannitol, high salinity and wounding (Feng *et al*., 2005; Sohn *et al*., 2006). In addition, *RAV1* is rapidly induced by cold and this response is regulated by the circadian clock (Fowler & Thomashow, 2002; Fowler *et al*., 2005). *Galegae orientalis* is a nitrogen-fixing legume used for forage production and soil improvement in scandinavian agriculture (Varis, 1986). Similarly to other plant species, *GoRAV* expression is induced by cold, exogenous ABA, high salinity and drought (Chen *et al*., 2009). Moreover, *BnaRAV-1-HY15*,

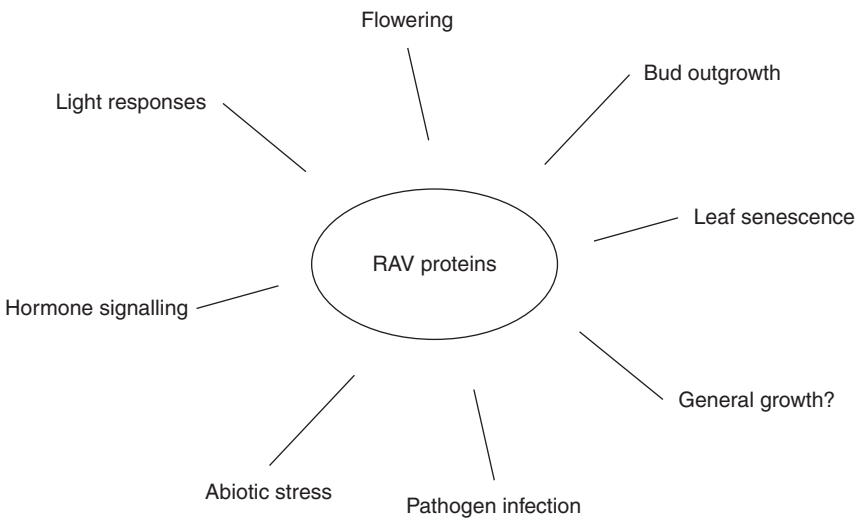

FIG. 3. Summary of the processes regulated by RAV proteins in different plant species.

a *RAV* orthologue in *Brassica napus*, an important agricultural-oil crop, is also induced by cold, NaCl and polyethylene glycol treatments (Zhuang *et al*., 2011).

A *RAV* orthologue (*CaRAV1*) from chili pepper (*Capsicum annuum*) is strongly induced during pathogen infection with *Xanthomonas campestris*, environmental stresses and abiotic elicitors (Sohn *et al*., 2006). Overexpression of *CaRAV1* in arabidopsis induces several PR genes and enhances resistance not only against other pathogens such as *Pseudomonas syringae*, but also against osmotic stresses by high dehydration and salinity (Sohn *et al*., 2006). *Solanum lycopersicum*, tomato, is the second most consumed vegetable in the world. *Ralstonia solanacearum* causes the bacterial wilt disease, probably the most important bacterial vascular disease in tomato (Hai *et al*., 2008). Ectopic expression of *SlRAV2* increases bacterial wilt tolerance in tomato plants by inducing the expression of PR genes such as *SlERF5* and *PR5* (Li *et al*., 2011).

Endogenous small RNA pathways and RNA silencing are major components of the plant response to different biotic and abiotic stresses. RNA silencing is a sequence-specific RNA degradation mechanism activated during viral infection that serves to protect plants against viruses (Ding and Voinnet, 2007). On the other side, plant viruses try to block the plant RNA silencing defence using different proteins (Diaz-Pendon and Ding, 2008). *TEM2* is essential for suppression of RNA silencing by at least two unrelated plant viral proteins, potyviral HC-Pro and carmoviral P38, two potent viral suppressors of silencing that block primary and transitive RNA silencing (Endres *et al*., 2010). In tobacco, both viral repressors require *NtRAV2* to block exclusively the activity of primary small interfering RNAs. *NtRAV2* interacts physically with HC-Pro proteins and is required for HC-Pro suppression of virus-induced gene silencing (VIGS). Moreover, *TEM2* induces the expression of *FRY1* and *CML38*, two genes that act as endogenous suppressors of silencing in arabidopsis (Anandalakshmi *et al*., 2000). Consequently, *TEM2* seems to be an essential control point in viral suppression of silencing. However, neither of the related arabidopsis genes *RAV1* or *TEM1* seems to have a redundant role in this specific aspect as they are not able to compensate for the loss of *TEM2* to divert host defences toward responses that interfere with antiviral silencing (Endres *et al*., 2010). *TEM2* may repress directly or indirectly the transcription of genes that encode proteins of the plant silencing machinery (Endres *et al*., 2010). Therefore, *RAV* orthologues from different plant species could function as key modulators of biotic and abiotic stress responses by integrating the regulation of diverse plant defence signalling pathways.

CONCLUSIONS

Despite *RAV* genes not being completely characterized, promising results obtained in recent years suggest strongly that RAV family members play important roles in many different physiological and developmental pathways in several plant species (Fig. 3). *RAV* genes act as repressors in the regulation of gene expression in various plant biological processes that may be of crucial importance for plant survival and crop production. Among these processes, floral transition is the best studied and *TEM1* and *TEM2* control at least the photoperiod- and GA-dependent flowering pathways. Moreover, *RAV* genes in different species may play important roles in other developmental processes and may also modulate some of the complex systems of response to diverse abiotic and biotic stresses. In conclusion, the RAV family, a unique family of transcription factors in plants, seems to integrate and control different physiological mechanisms that are affected by many internal and external factors. These essential controls should contribute to improve plant fitness, with the final outcome being optimal plant development and adaptation to environmental threats.

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