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Temperature-dependent control of flowering time in barley (*Hordeum vulgare* L.) by the gibberellin signaling pathway

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SUMMARY

The mechanism underlying the regulation of flowering time by the phytohormone gibberellin (GA) in the context of cold temperature is poorly understood. In *Arabidopsis*, low temperature delays growth and flowering. At the molecular level this delay can be explained by the fact that cold temperature promotes the catabolism of GA, leading to an accumulation of DELLA proteins, the major repressors of GA responses (Achard *et al.*, 2008a; Schwechheimer, 2012). DELLA abundance responds to changes in temperature, and the effect of DELLA accumulation can be suppressed by GA treatments. In *Arabidopsis*, barley (*Hordeum vulgare*) and rice (*Oryza sativa*) GA has a very important role in the regulation of flowering time: GA biosynthesis mutants in these species show a strong delay in flowering. In *Arabidopsis*, the MADS-box transcription factor APETALA1 (AP1) is well known to play a pivotal role in determining the floral meristem identity and its expression is downstream the flowering promoting pathways (Mandel and Yanofsky, 1995). Moreover, it has been shown in our laboratory that AP1 is directly repressed by DELLAs. In barley, VERNALIZATION1 (VRN1), the closest homologue of AP1, is the master regulator of flowering time (Distelfeld *et al.*, 2009). In winter varieties *VRN1* expression is gradually induced with exposure to cold temperature, a process known as vernalization, whereas in spring varieties the expression of *VRN1* independent from a cold stimulus is the basis for their vernalization-independent flowering. I can thus hypothesize that DELLA protein from barley represses flowering through interactions with AP1/VRN1, and this repression is relieved (i) by the GA-dependent DELLA degradation and (ii) by increased activity of AP1/VRN1. I want to understand how flowering time in barley responds to temperature and gibberellin. In cold temperature I could see a delay in flowering, and this delay could be rescued by GA application or with an increased VRN1 activity. Probably, the promoting effect of GA on flowering time is linked to an increase of *VRN1* expression, following a GA treatment. Moreover, I want to understand if there is a correlation between the delay in flowering in barley at low temperature and the effect of GA and temperature with the expression levels of the genes involved in GA synthesis and deactivation. It seems to exist a correlation between the expression levels of the GA biosynthesis genes and the time to flower, in normal as well as in cold temperature growth conditions. More in detail, I want to understand if the GA biosynthesis genes in barley are subjected to cold and GA regulation (Hedden and Phillips, 2000; Olszewski *et al.*, 2002). In barley, cold temperature and applied GA promote the activity of the genes involved in the deactivation of GA, leading to a probable decrease in the endogenous GA content, affecting then the flowering time. I want also to understand if the AP1/DELLA interaction identified in *Arabidopsis* also takes place between VRN1 and SLENDER1 (SLN1), the only DELLA protein from barley, and if the flowering time control in this species, in response to gibberellin and temperature, is dependent on this interaction.

ZUSAMMENFASSUNG

Der Mechanismus, der der Regulierung der Blütezeit durch das Phytohormon Gibberellin (GA) im Zusammenhang mit niedriger Temperatur zu Grunde liegt, ist wenig verstanden. In *Arabidopsis* verzögert niedrige Temperatur Wachstum und Blüte. Auf molekularer Ebene kann diese Verzögerung durch die Tatsache erklärt werden, dass die kalte Temperatur den GA-Katabolismus fördert, was zu einer Akkumulation von DELLA-Proteinen, den Hauptrepressoren der GA-Reaktionen, führt (Achard et al., 2008a; Schwechheimer, 2012). Die DELLA-Abundanz reagiert auf Temperaturänderungen, und die Wirkung der DELLA-Akkumulation kann durch GA-Behandlung unterdrückt werden. In *Arabidopsis*, Gerste (*Hordeum vulgare*) und Reis (*Oryza sativa*) spielt GA eine sehr wichtige Rolle bei der Regulierung der Blühzeit: GA-Biosynthese-Mutanten in diesen Spezies zeigen eine starke Verzögerung der Blüte. In *Arabidopsis* ist bekannt, dass der MADS-Box-Transkriptionsfaktor APETALA1 (AP1) eine entscheidende Rolle bei der Festlegung der Blütenmeristem-Identität spielt und seine Expression ist *downstream* der Signalwege, die die Blühinduktion fördern (Mandel und Yanofsky, 1995). Darüber hinaus wurde in unserem Labor gezeigt, dass AP1 direkt von DELLAs unterdrückt wird. In Gerste ist VERNALIZATION1 (VRN1), das engste Homolog von AP1, der Hauptregulator der Blühzeit (Distelfeld et al., 2009); in Winter-Sorten wird seine Expression allmählich durch Einwirkung niedriger Temperaturen induziert, ein Prozess, der als Vernalisation bekannt ist, wohingegen in Frühlingssorten die Expression von VRN1 Kälte-unabhängig die Grundlage für ihre vernalisationsunabhängige Blüte ist. Ich kann daher die Hypothese aufstellen, dass das DELLA-Protein aus Gerste das Blühen durch Wechselwirkungen mit AP1/VRN1 reprimiert, und diese Repression durch (i) den GA-abhängigen DELLA-Abbau und (ii) durch erhöhte Aktivität von AP1/VRN1 gelindert wird. Ich möchte verstehen, wie die Blühzeit in Gerste auf Temperatur und Gibberellin reagiert. Bei niedriger Temperatur konnte ich eine Verzögerung der Blüte beobachten, und diese Verzögerung konnte durch GA-Zugabe oder mit einer erhöhten VRN1-Aktivität gerettet werden. Wahrscheinlich ist die fördernde Wirkung von GA auf die Blühzeit mit einer Erhöhung der *VRN1*-Expression nach einer GA-Behandlung verbunden. Darüber hinaus möchte ich verstehen, ob es eine Korrelation zwischen der Verzögerung der Blüte in Gerste bei niedriger Temperatur und der Wirkung von GA und Temperatur mit den Expressionsniveaus der Gene gibt, die an der GA-Synthese und -Deaktivierung beteiligt sind. Es scheint eine Korrelation zwischen den Expressionsniveaus der GA-Biosynthesegene und der Zeit bis zur Blüte, sowohl bei normalen als auch bei niedrigen Temperaturbedingungen, zu bestehen. Genauer gesagt möchte ich verstehen, ob die GA-Biosynthesegene in Gerste der Kälte- und GA-Regulierung unterliegen (Hedden und Phillips, 2000; Olszewski et al., 2002). In Gerste fördern kalte Temperatur und zugegebene GA die Aktivität der Gene, die an der Deaktivierung von GA beteiligt sind, was wahrscheinlich zu einer Abnahme des endogenen GA-Gehalts führt, und infolgedessen den Blühzeitpunkt beeinflusst. Ich möchte auch verstehen, ob die in *Arabidopsis* identifizierte AP1/DELLA-Interaktion auch zwischen VRN1 und

SLENDER1 (SLN1) stattfindet, dem einzigen DELLA-Protein aus Gerste, und ob die Blühzeitkontrolle in dieser Spezies als Reaktion auf Gibberellin und Temperatur abhängig von dieser Interaktion erfolgt.

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LIST OF ABBREVIATIONS

AGL24	*AGAMOUS-LIKE24*
AP1	*APETALA1*
AP2	*APETALA2*
AP3	*APETALA3*
BiFC	Bimolecular Fluorescence Complementation Assay
CAL	*CAULIFLOWER*
Co-IP	Co-Immunoprecipitation
CO	*CONSTANS*
$SCF^{SLY1/GID2}$	E3 Ubiquitin ligase complex
FM	Floret Meristem
FLC	*FLOWERING LOCUS C*
FLM	*FLOWERING LOCUS M*
FT	*FLOWERING LOCUS T*
FUL	*FRUITFULL*
GID1	*GA INSENSITIVE DWARF1*
GID2	*GA INSENSITIVE DWARF2*
GRAS	GAI, RGA, SCR
GAI	*GIBBERELLIC ACID INSENSITIVE*
GA(s)	Gibberellin(s)
Grd5	*GIBBERELLIN-RESPONSIVE DWARF 5*
LFY	*LEAFY*
OGP	Outer Glume Primordia
Ppd-H1	*Photoperiod-H1*
PI	*PISTILLATA*
RGA	*REPRESSOR OF ga1-3*
RGL	*RGA-LIKE*
SCR	*SCARECROW*
SEP3	*SEPALLATA3*
SVP	*SHORT VEGETATIVE PHASE*
SLY1	*SLEEPY1*
SLR1	*SLENDER RICE1*
SLN1	*SLENDER1*
SNZ	*SNEEZY*
SM	Spikelet Meristem
STM	Spikelet Triplet Meristem
SPL	*SQUAMOSA PROMOTER BINDING PROTEIN-LIKE*
SOC1	*SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1*
TFL1	*TERMINAL FLOWER1*
VRN1	*VERNALIZATION1*
VRN2	*VERNALIZATION2*
VRN3	*VERNALIZATION3*
Y2H	Yeast two hybrid assay

INTRODUCTION

1. The gibberellin signaling pathway

Gibberellins (GAs) are plant hormones essentials for the regulation of several developmental processes: seed germination, stem elongation, leaf expansion, trichome development, and pollen maturation. They also promote the transition from the vegetative to reproductive phase, with an important role in flower development and flowering induction (Fleet and Sun, 2005; Pimenta-Lange and Lange, 2006; Achard and Genschik, 2009).

Gibberellin biosynthesis and deactivation mechanisms are finely controlled in plants, for the pivotal role of GAs in the regulation of the development of organs and tissues in response to changes in the environment. In plants, more than one hundred GAs have already been identified but only few of them are precursors of the bioactive forms or deactivate metabolites (Yamaguchi, 2008). The amount of biologically active GAs in plants is determined by the ratio between synthesis and deactivation. GA biosynthesis in higher plants can be divided in three steps (Figure 1): (i) biosynthesis of *ent*-kaurene in proplastids, (ii) conversion of *ent*-kaurene in GA_{12} via microsomal cytochrome P450 monooxygenases in the ER membrane, and (iii) formation of C_{20}- and C_{19}- GAs in the cytosol (Olszewski *et al.*, 2002). During the third step occurs the formation of the bioactive GAs. This step is under the regulation of three classes of enzymes, localized in the cytosol: GA20ox, GA3ox and GA2ox. The GA20ox and GA3ox enzymes are required for the formation of precursors of bioactive GA, and for the activation of the precursors into bioactive forms, respectively. The GA2ox enzymes are required for the deactivation of the bioactive GA, and for the depletion of the precursors of active GA, otherwise converted into bioactive forms. The overexpression of these genes can alter the concentrations of the bioactive GAs, indicating that the regulation of these genes has a key role in modulating the amount of GAs present in plants (Hedden and Phillips, 2000; Yamaguchi and Kamiya, 2000). Moreover, at least in *Arabidopsis*, these genes are subjected to a feedback regulation by GA, required to control the endogenous concentration of bioactive GA in plants. Most of the *GA20ox* and *GA3ox* genes are downregulated by applied GA, whereas the *GA2ox* genes are upregulated by GA treatments (Thomas *et al.*, 1999; Hedden and Phillips, 2000; Yamaguchi and Kamiya, 2000).

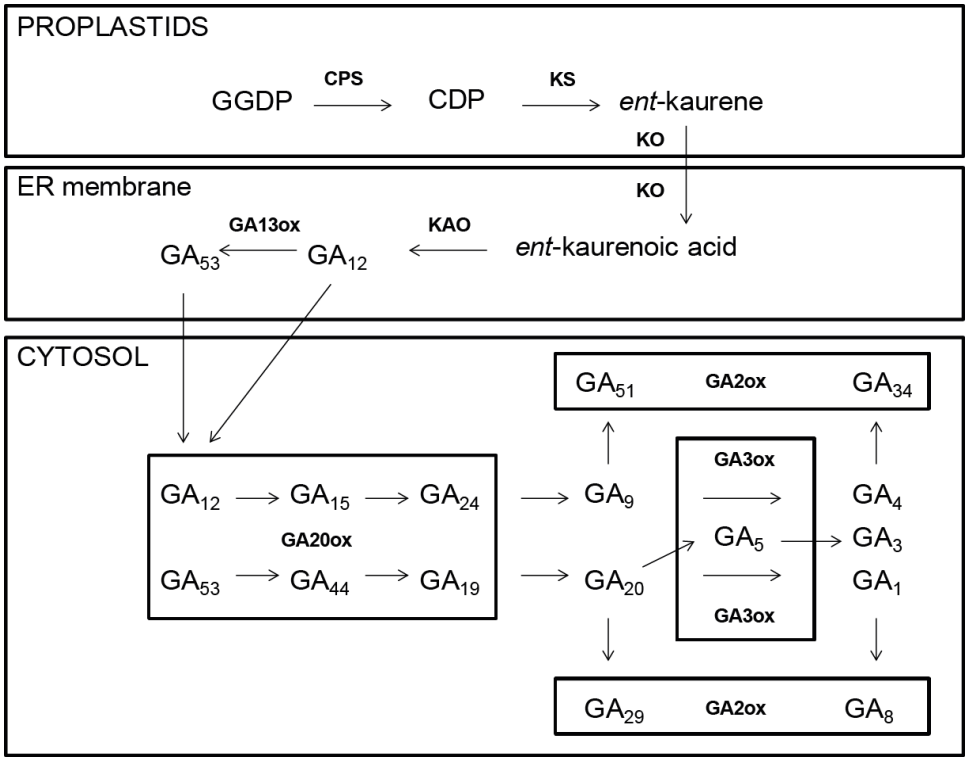

Figure 1. Gibberellin biosynthesis and deactivation pathways. The GA20ox enzymes catalyze the limiting steps of the pathway. GA_8, GA_{29}, GA_{34}, and GA_{51} are the inactive catabolites; whereas GA_1, GA_3, and GA_4 are the bioactive forms. GGDP, geranylgeranyl diphosphate; CPS, *ent*-copalyl diphosphate synthase; CDP, *ent*-copalyl diphosphate; KS, *ent*-kaurene synthase; KO, *ent*-kaurene oxydase; ER, endoplasmic reticulum; KAO, *ent*-kaurenoic acid oxydase; GA13ox, GA 13-oxydase; GA20ox, GA 20-oxydase; GA3ox, GA 3-oxydase; GA2ox, GA 2-oxydase.

1.1. The DELLA proteins

The GRAS protein family is specific for plants, and the name derives from the three first-identified members: GAI, RGA, SCARECROW (SCR), (Di Laurenzio *et al.*, 1996; Peng *et al.*, 1997; Silverstone *et al.*, 1998). All GRAS proteins share a C-terminal domain, which is highly conserved and involved in the transcriptional regulation. This domain is characterized by the presence of two leucine rich repeats, LHRI and LHRII, and three motifs, VHIID, PFYRE and SAW (Bolle *et al.*, 2004). DELLA proteins are part of this family, and can be distinguished from other GRAS proteins for the presence of a specific N-terminal sequence, containing two conserved domains: the DELLA domain and the TVHYNP domain (Figure 2). These proteins are highly conserved among *Arabidopsis*, barley, maize, rice and wheat (Peng *et al.*, 1997; Ikeda *et al.*, 2001; Chandler *et al.*, 2002; Van De Velde *et al.*, 2017).

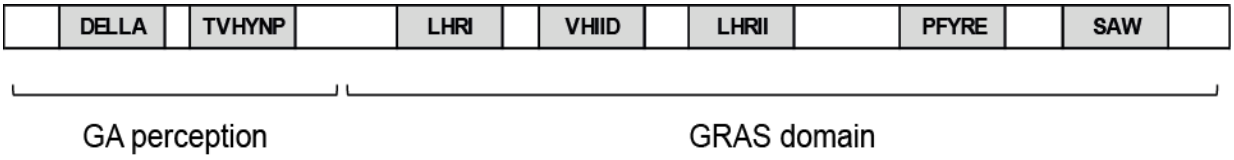

Figure 2. General structure of a DELLA protein. The DELLA and TVHYNP motifs located at the GA perception domain at the N-terminus of the protein are required for the GA perception, for the interaction with GID1, and transactivation activity. The GRAS domain carries the LHRI motif, required for the dimerization; the VHIID and LHRII motifs, required for the interaction with SLY and protein degradation; the PFYRE and SAW motifs, required for growth repression.

DELLAs are well known as key repressors of the GA-dependent process, and the presence of GA relieves their repressor activity (Achard and Genschik, 2009). Subsequently, the lack of DELLA function suppresses the GA deficient phenotype (Dill and Sun, 2001; King *et al.*, 2001; Lee *et al.*, 2002; Cheng *et al.*, 2004; Tyler *et al.*, 2004). Five DELLA proteins, with distinct and overlapping functions, have been identified in *Arabidopsis*: RGA, GAI, RGL1, RGL2, and RGL3. RGA and GAI are the major repressors of vegetative growth and floral induction (Richards *et al.*, 2001; Olszewski *et al.*, 2002; Achard *et al.*, 2008b). RGL2 alone plays an important role in seed germination (Lee *et al.*, 2002), whereas together with RGA and RGL1 regulates the flower development (Richards *et al.*, 2001; Cheng *et al.*, 2004; Tyler *et al.*, 2004; Achard *et al.*, 2008b). RGL3 has a role in the modulation of environmental stress (Wild *et al.*, 2012). In barley and rice only one DELLA has been found, SLENDER1 (SLN1) and SLENDER RICE1 (SLR1), respectively, and both are involved in the repression of the GA-dependent responses.

GA signaling mutants can be distinguished in two different categories, which have been characterized across several different plant species. The first category shows a partially dominant GA-insensitive (or GA-non responsive) phenotype. These mutants fail to grow more rapidly in response to applied GA. However, when they are further dwarf for genetic reason or by application of GA biosynthesis inhibitors (Koornneef *et al.*, 1985; Winkler and Freeling, 1994), a growth response to exogenous GA is observed but is limited only back to that of the original dwarf (Winkler and Freeling, 1994). To this category belong wheat (*Triticum aestivum*, *Rht*; Gale and Marshall, 1973), maize (*Zea mays*, *D8*; Phinney, 1956) and *Arabidopsis* (*gai*; Koornneef *et al.*, 1985). The second category, named slender mutants, exhibits an extremely rapid growth, and a constitutive GA response. Slender mutants have been identified in barley (*Hordeum vulgare*; Foster, 1977), pea (*Pisum sativum*; Potts *et al.*, 1985) and rice (*Oryza sativa*; Ikeda *et al.*, 2001), and all show rapid growth even in GA-deficient background, or when treated with inhibitors of GA biosynthesis (Crocker *et al.*, 1990; Ikeda *et al.*, 2001). For these reasons, the growth of slender plants is independent or requires much lower than normal of bioactive GA. The slender phenotype is recessive and represents a loss of function, then the wild type *SLENDER* gene product, encoded by the *Slender1* (*Sln1*) locus in barley, is a negative regulator of GA-regulated responses, through

which GA signaling proceeds (Chandler and Robertson, 1999). In *Arabidopsis*, DELLA proteins repress GA responses, and the degree of repression is modulated by GA signaling. In wild type plants, a high content of bioactive GA promotes GA signaling, blocks DELLA action and growth is rapid. In a GA-deficient mutant, the low amount of bioactive GA results in a reduction of GA signaling, repression by DELLAs remain high and growth is slow. In barley and rice, *della* mutants show two radically different phenotypes, and each is the result of a single nucleotide substitution in the *Sln1* gene. One phenotype is the highly elongated 'slender' types, named 'slender DELLA'. It is recessive, shows male sterility, and an extreme GA response. The other one is the GA-insensitive dwarf, named 'dwarf DELLA'. It is dominant, fully fertile and does not respond to GA treatment. (Ikeda *et al.*, 2001; Chandler *et al.*, 2002; Asano *et al.*, 2009; Chandler and Harding, 2013).

1.2. Gibberellin perception and DELLA degradation

In absence of gibberellins, DELLA proteins accumulate and repress the GAs developmental responses. In presence of GAs there is the formation of the GA-GID1-DELLA complex, which stimulates the DELLAs degradation through the recognition of the DELLAs by SLY1. This recognition triggers DELLA ubiquitinylation and the subsequent degradation by the 26S proteasome.

The binding of bioactive GA with the N-terminal domain of the soluble receptor GA-INSENSITIVE DWARF1 (GID1) leads to conformational changes in the receptor, promoting the interaction between DELLA and GID1 (Nakajima *et al.*, 2006; Ueguchi-Tanaka *et al.*, 2007). The DELLA and the TVHYNP domains of DELLA proteins are required for the interaction of DELLA with GID1, thus deletions of these regions do not lead the DELLA-GID1 interaction, even in presence of GA (Griffiths *et al.*, 2006; Willige *et al.*, 2007). Once the GID1-GA-DELLA complex is formed, the interaction between DELLA and the E3 ubiquitin ligase, $SCF^{SLY1/GID2}$, can occur (Dill *et al.*, 2004; Fu *et al.*, 2004; Hirano *et al.*, 2010; Ariizumi *et al.*, 2011). This binding causes the ubiquitinylation and subsequent degradation of DELLAs through the 26S proteasome (Viestra, 2009).

The GA-induced and proteasome-mediated degradation of DELLA repressor proteins was first observed by the stabilization of the barley protein SLN1, after the use of proteasome inhibitors (Silverstone *et al.*, 2001; Fu *et al.*, 2002). These results were confirmed by the further identification of two *Arabidopsis* F-box proteins, SLEEPY1 (SLY1) and SNEEZY (SNZ), and their rice orthologue, GA INSENSITIVE DWARF2 (GID2) (McGinnis *et al.*, 2003; Sasaki *et al.*, 2003). The *sly1* and the *gid2* mutants were the first examples of mutants that reflect the full spectrum of GA-associated, recessive phenotypes (Steber *et al.*, 1998; Sasaki *et al.*, 2003; Strader *et al.*, 2004). F-box proteins constitute subunits of the E3 ubiquitin ligase SCF (SKP1-CULLIN-F-BOX) complexes,

and their task is the polyubiquitinylation of the F-box protein-specific substrates for the 26S proteasome-mediated degradation (Vierstra, 2009).

2. Flowering time

The transition from vegetative to reproductive development - the floral transition - is the most dramatic phase change in plant development, and a critical step for a successful sexual reproduction. This transition is under the control of a complex genetic network that monitors the developmental state of the plant as well as the surrounding environment, to ensure the correct timing of flowering when both internal and external conditions are appropriate. Temperature and day length have the strongest influence on flowering, and the ability of the plant to perceive and respond to these signals is controlled by the vernalization pathway and the photoperiod pathway.

2.1. Flowering time regulation in *Arabidopsis thaliana*

The regulation of flowering time has been studied for more than 100 years. The molecular mechanisms involved have been investigated in different flowering plants but mainly in *Arabidopsis thaliana*. Genetic analysis of mutants impaired in the correct timing of flowering has identified about 80 genes, placed in multiple genetic pathways, controlling the floral transition. The correct timing for flowering is the result of the integration of exogenous and endogenous signals, and five distinct but interconnected pathways, involved in the control of this process, have been described (Figure 3). Vernalization accelerates flowering upon a several weeks of exposure to low temperatures (Koornneef *et al.*, 1998; Simpson *et al.*, 1999; Reeves and Coupland, 2000; Samach and Coupland, 2000; Araki, 2001; Mouradov *et al.*, 2002; Jarillo and Piñero, 2011; Srikanth and Schmid, 2011; Andrés and Coupland, 2012). Photoperiod, the duration of the daily light period, through the control of the circadian rhythm, regulates the timing to flower on the basis of the day length and light quality (Koornneef *et al.*, 1998; Simpson *et al.*, 1999; Reeves and Coupland, 2000; Samach and Coupland, 2000; Araki, 2001; Mouradov *et al.*, 2002; Jarillo and Piñero, 2011; Srikanth and Schmid, 2011; Andrés and Coupland, 2012). The autonomous pathway was identified through the analysis of mutants with a late flowering phenotype in all the photoperiods, and very responsive to vernalization (Simpson *et al.*, 1999; Araki, 2001; Mouradov *et al.*, 2002; Srikanth and Schmid, 2011). Genes involved in GA synthesis and signal transduction have been suggested to form a distinct pathway required for a normal flowering time (Koornneef *et al.*, 1998; Simpson *et al.*, 1999; Araki, 2001; Mouradov *et al.*, 2002; Srikanth and Schmid, 2011; Conti, 2017). Finally, age is an endogenous input whose role in flowering has been described only some years ago (Jarillo and Piñero, 2011; Srikanth and Schmid, 2011).

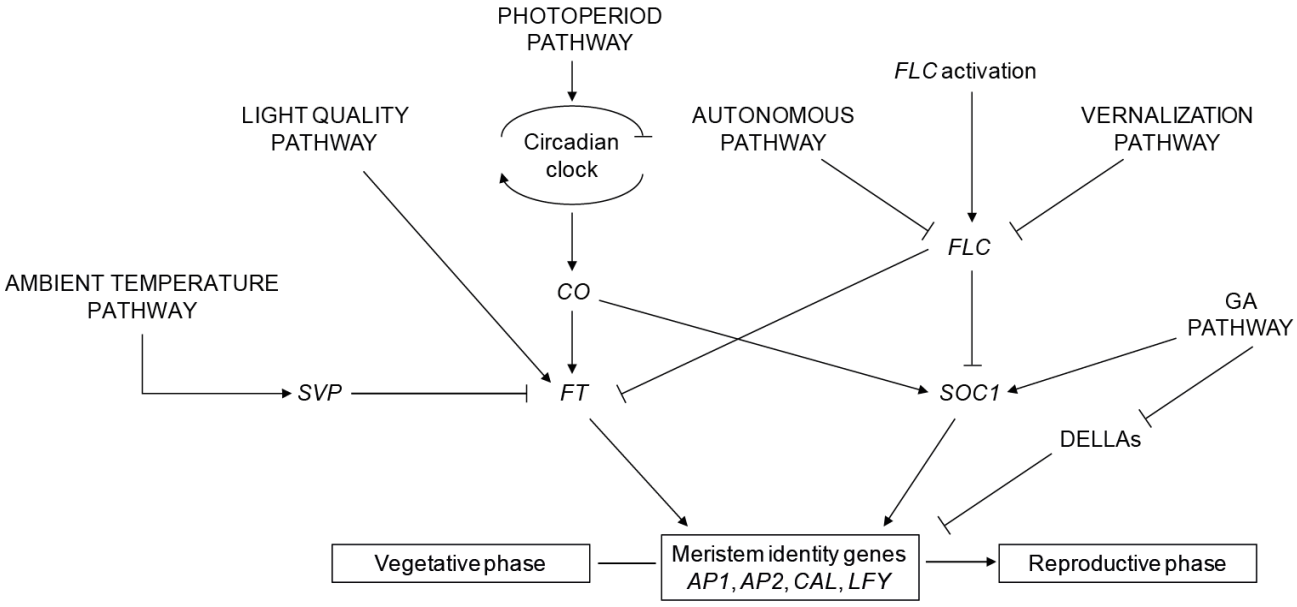

Figure 3. Flowering time control in *Arabidopsis thaliana*. All the known pathways converge in the activation of the meristem identity genes. *APETALA1* (*AP1*), *APETALA2* (*AP2*), *CAULIFLOWER* (*CAL*), *CONSTANS* (*CO*), *FLOWERING LOCUS C* (*FLC*), *FLOWERING LOCUS T* (*FT*), *LEAFY* (*LFY*), *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS* (*SOC1*), *SHORT VEGETATIVE PHASE* (*SVP*).

2.1.1. The gibberellin pathway and its role in flowering time regulation

The gibberellin pathway has an important role in the regulation of flowering in dicots, like *Arabidopsis*, and in monocots, like barley, controlling the transition from the juvenile to the adult growth phase. The role of GA in this transition is due to its function as integrator of several different environmental and endogenous signals through the DELLA-mediated pathway (Davière *et al.*, 2008).

In *Arabidopsis*, exogenous application of gibberellin promotes flowering (Langrige, 1957), and several mutations affecting the GA biosynthesis pathway have been identified. The importance of GAs is clearly illustrated by the *Arabidopsis ga1-3* mutant. This mutant contains a large deletion in the *GA1* gene, which encodes *ent*-copalyl diphosphate synthase, the enzyme catalyzing the first committed step in GA biosynthesis, thus this mutation provides a block in the GA production (Sun *et al.*, 1992; Sun and Kamiya, 1994). The large reduction in bioactive GAs in *ga1-3* leads to a GA-deficient phenotype characterized by dark green leaves and severe dwarfism (Koornneef and van der Veen, 1980; Silverstone *et al.*, 2001). The *ga1-3* plant is also impaired in root growth and trichome initiation, and it shows reduced apical dominance (Chien and Sussex, 1996; Silverstone *et al.*, 1997; Fu and Harberd, 2003). Under inductive long day conditions, floral initiation in *ga1-3* is delayed and flowers are male sterile (Koornneef *et al.*, 1983; Wilson *et al.*, 1992). In short day conditions, however, *ga1-3* remains vegetative until it eventually undergoes senescence without

flowering (Wilson *et al.*, 1992). Also, *ga1-3* seeds cannot germinate without exogenous GA applications (Koornneef *et al.*, 1983). In contrast, *ga4* and *ga5* mutants, defective in GA3ox and GA20ox activity, respectively, have less severe effects, and the semi-dwarf plants are able to produce fertile flowers (Koornneef and van der Veen, 1980; Talon *et al.*, 1990).

Loss-of-function mutations in *RGA* and *GAI* can suppress some of the effects of GA deficiency, suggesting that RGA and GAI negatively regulate a subset of GA responses in *Arabidopsis* (Peng *et al.*, 1997; Silverstone *et al.*, 1997), functioning as transcriptional regulators that directly or indirectly repress the expression of GA-induced genes (Tyler *et al.*, 2004). The *rga-28* mutation in the Col-0 background restored petal development, and also slightly rescued the stamen defect and male infertility of *ga1-3* (Tyler *et al.*, 2004). In contrast, *rga* alleles in the Ler background have no effect on the floral defect or male sterility of *ga1-3* (Silverstone *et al.*, 1997; Dill and Sun, 2001). The *erecta* (*er*) mutation in Ler enhances dwarf phenotypes of GA-deficient or GA-insensitive mutants (Fridborg *et al.*, 2001). A loss-of-function *rga* mutation may more readily suppress the floral defect of *ga1-3* in the Col-0 ecotype than in the Ler background, because Col-0 does not contain the *er* mutation (Tyler *et al.*, 2004). The *rgl1* and *rgl2* mutants, in combination with *rga*, significantly increase the stamen filament growth, anther development, and fertility of *ga1-3* flowers (Tyler *et al.*, 2004). However, the quadruple mutant *ga1*/*rga*/*rgl1*/*rgl2* still does not reach wild-type levels of fertility. Thus, there is a high degree of functional redundancy in controlling flower development. (Tyler *et al.*, 2004). Because the *er* mutation is in itself a dwarfing mutation, strongly affecting inflorescence elongation and organ shape, and because almost all of the GA mutant dwarfs have been isolated in the *er* background, it is probable that *er* might influence the phenotypic effects of mutations in the reductions in GA levels or responses (Fridborg et al., 2001).

Expression of *GA20ox* is regulated by environmental and physiological changes, and increases in long days: therefore its high expression level correlates with conditions that induce early flowering (Xu *et al.*, 1997). Moreover, transgenic plants with an elevated content of GA_4 flower earlier than wild type plants, both in long and short days (Huang *et al.*, 1998; Coles *et al.*, 1999). This suggests that GA levels are limiting for flowering time, and this is consistent with previous observations that GA application causes early flowering of wild type plants.

Mutants impaired in GA biosynthesis (for example *ga1*, defective in the early steps of GA production) are moderately late flowering under long day conditions but do not flower under short day conditions (Wilson *et al.*, 1992). These data suggest that the GA pathway and the long day pathway work in parallel. Moreover, the *ga1* mutant in combination with a mutation which alters the long day pathway, for example the *co* mutant, produces double mutants that are not able to flower in long day conditions (Putterill *et al.*, 1995). These results lead to the conclusion that in short days,

where the long day pathway is not active, the GA pathway has a predominant role in the control of flowering and when its role fails, flowering is blocked. Conversely, in long days, where the long day pathway is active, the inactivation of the GA pathway does not have such severe consequences (Putterill *et al.*, 1995; Reeves and Coupland, 2000; Mouradov *et al.*, 2002; Jarillo and Piñero, 2011). These phenotypic observations indicate an absolute requirement for GAs when the photoperiodic pathway is not active. They also suggest that GAs production is largely dispensable under long day conditions, presumably as a result of the activation of the photoperiodic pathway and consequent mobilization of FT (FLOWERING LOCUS T) in the apex (Conti, 2017).

Gibberellins regulate the expression of *SOC1* (*SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1*) and *LFY* (*LEAFY*) in a positive manner and together with *SOC1* and *FT*, GAs regulate the expression of flower meristem identity genes *AP1* (*APETALA1*), *APETALA2* (*AP2*) and *CAULIFLOWER* (*CAL*) (Blázquez and Weigel, 2000; Moon *et al.*, 2003).

SOC1 has an important role in the integration of GA-dependent flowering pathways, particularly for flowering under short days. The *ga1-3* mutant does not flower under short days, and its expression of *SOC1* is very low, and GA treatments lead *ga1-3* to flower at a similar time to that for GA-treated wild type plants, and to have a *SOC1* expression similar to that in GA-treated wild type plants (Moon *et al.*, 2003). Moreover, flowering is delayed in *gai-1* mutants irrespective of GA treatments and the level of *SOC1* is minimal, demonstrating a correlation between flowering time and a low *SOC1* expression, as a result of a defect in the gibberellin signaling pathway (Moon *et al.*, 2003). Additionally, the introduction of *SOC1* overexpression into *ga1-3* can rescue the non-flowering phenotype under short days. Taken together, these results suggest that the failure of flowering by GA-deficient mutants under short days is caused by the lack of *SOC1* activation, and that *SOC1* has a central role in the integration of the GA-dependent flowering pathways (Moon *et al.*, 2003).

Although *SOC1* is regulated by GA and its expression level correlate with flowering time, as described above, it is not the only flowering time regulator controlled by GA. *LFY*, one of the flower meristem identity gene, is another important factor regulated by GA. In *Arabidopsis*, flowering can be abolished by simultaneous inactivation of photoperiod and gibberellin pathways, as demonstrated by the analysis of the *co ga1* double mutant (Putterill *et al.*, 1995) and GAs promote flowering by increasing the transcriptional activity of *LFY*, the floral meristem identity gene. In *co ga1* mutants, *LFY* levels are very low, in contrast with the upregulation of *LFY* in wild type plants (Blázquez and Weigel, 2000). Similarly, expression of *LFY::GUS* is reduced in mutants defective in GA biosynthesis, and increases in mutants with constitutive GA signaling, confirming the idea that the photoperiod and the gibberellin pathways converge upstream of *LFY*. Moreover, overexpression of *LFY* restores flowering of *ga1-3* mutants in short days (Blázquez *et al.*, 1998).

These results suggest that GA regulates in an independent manner *LFY* and *SOC1*, the two flowering pathway integrators. And, obviously, the presence of additional factors in the regulation of flowering time in response to GA cannot be excluded.

2.1.2. The MADS-box transcription factor *APETALA1* (*AP1*)

In *Arabidopsis*, *APETALA1* (*AP1*) encodes a MADS-box transcription factors well known to confer floral meristem identity to the shoot apical meristem: it regulates flower development together with *APETALA3* (*AP3*), *PISTILLATA* (*PI*), and *SEPALLATA3* (*SEP3*) (Mandel *et al.*, 1992; Mandel and Yanofsky, 1995). *AP1* expression is first observed in emerging floral primordia and, later on, is confined to the outer whorls of flower buds, from which sepals and petal arise (Mandel *et al.*, 1992). During early flower development, AP1 represses the expression of the flowering time genes *AGL24* (*AGAMOUS-like 24*) and *SOC1* (Liu *et al.*, 2007). It also represses the shoot identity gene *TFL1* (*TERMINAL FLOWER 1*), promoting the expression of *LFY* and controlling the expression of the floral homeotic genes (Liljegren *et al.*, 1999; Ng and Yanosfky, 2001; Gregis *et al.*, 2009). Mutations in the *AP1* gene cause the conversion of sepals, located in the first whorl, into leaf-like structures, which often develop secondary flowers in their axils. Moreover, this pattern can be repeated, and the axillary secondary flowers produce tertiary flowers in the axils of their first whorl. The formation of secondary flowers can be interpreted as a partial reversion of the floral meristem into an inflorescence meristem. Furthermore, the second whorl fails to develop correctly and the *ap1* mutant flowers do not form petals. Stamens and carpels, which originate, respectively, from the third and fourth whorls, are normal (Mandel *et al.*, 1992; Mandel and Yanofsky, 1995).

The constitutive expression of *AP1* alters not only inflorescence meristem identity, but also affects the vegetative phase of plants. During this phase, before the transition to the reproductive development, a rosette of leaves is produced. The constitutive expression of *AP1* influences the behavior of the vegetative meristem in continuous light as well as in short day conditions: plants which ectopically express *AP1* show a reduction in flowering time in comparison to wild type plants and, moreover, the reduction of the time to flower is much stronger in short days (Mandel and Yanofsky, 1995).

2.2. Flowering time regulation in cereal crops and barley

The adaptability of barley to many different environments is due to its allelic diversity in the *VERNALIZATION* (*VRN*) genes, regulating the vernalization response (Danyluk *et al.*, 2003; Trevaskis *et al.*, 2003; Yan *et al.*, 2003). The differences in the *VRN* genes lead to the division of barley into winter and spring varieties: winter varieties require long exposure to cold in order to flower, the so-called vernalization requirement, whereas spring varieties flower without exposure to low temperatures (Danyluk *et al.*, 2003; Trevaskis *et al.*, 2003; Yan *et al.*, 2003; Szücs *et al.*, 2007; Hemming *et al.*, 2009).

2.2.1. The vernalization requirement in cereals

The vernalization requirement is a very important agronomic trait, used to extent the plant growing time, increasing then the plant size and seed yield.

VRN1 encodes a MADS-box transcription factor with high similarity to *AP1*, *CAL* and *FUL* (*FRUITFUlL*) from *Arabidopsis thaliana* (Figure 4), and it has a key role in the control of the transition from vegetative to reproductive phase of the shoot apical meristem (Danyluk *et al.*, 2003; Trevaskis *et al.*, 2003; Yan *et al.*, 2003, 2004a). In diploid wheat, the activity of *VRN1* is essential for flowering: a mutant with a deletion in *VRN1* or a deletion of genes flanking *VRN1* is unable to flower (Shitsukawa *et al.*, 2007; Distelfeld and Dubcovsky, 2010).
The *VRN2* region contains two *ZCCT* genes, which encode putative zinc finger and CCT domain-containing proteins, without clear homologs in *Arabidopsis* (Yan *et al.*, 2004b). The *VRN2* region acts as a repressor of flowering and deletion of these two genes is associated with a spring growth habit in barley and in wheat (Yan *et al.*, 2004b; Dubcovski *et al.*, 2005; von Zitzewitz *et al.*, 2005; Distelfeld *et al.*, 2009).
VRN3 encodes a protein very similar to *FT* from *Arabidopsis*, which is known to be a long-distance flowering signal, that moves from leaves to apices inducing the meristem identity genes (Yan *et al.*, 2006; Corbesier *et al.*, 2007; Tamaki *et al.*, 2007; Turk *et al.*, 2008). The role of FT homologues in temperate cereals seem to be similar to the one described for *Arabidopsis*. Transgenic wheat plants overexpressing TaFT show a parallel increase in VRN1 transcript, suggesting a transcriptional activation of VRN1 by TaFT possibly through interactions with TaFDL2 protein (Li and Dubcovsky, 2008).

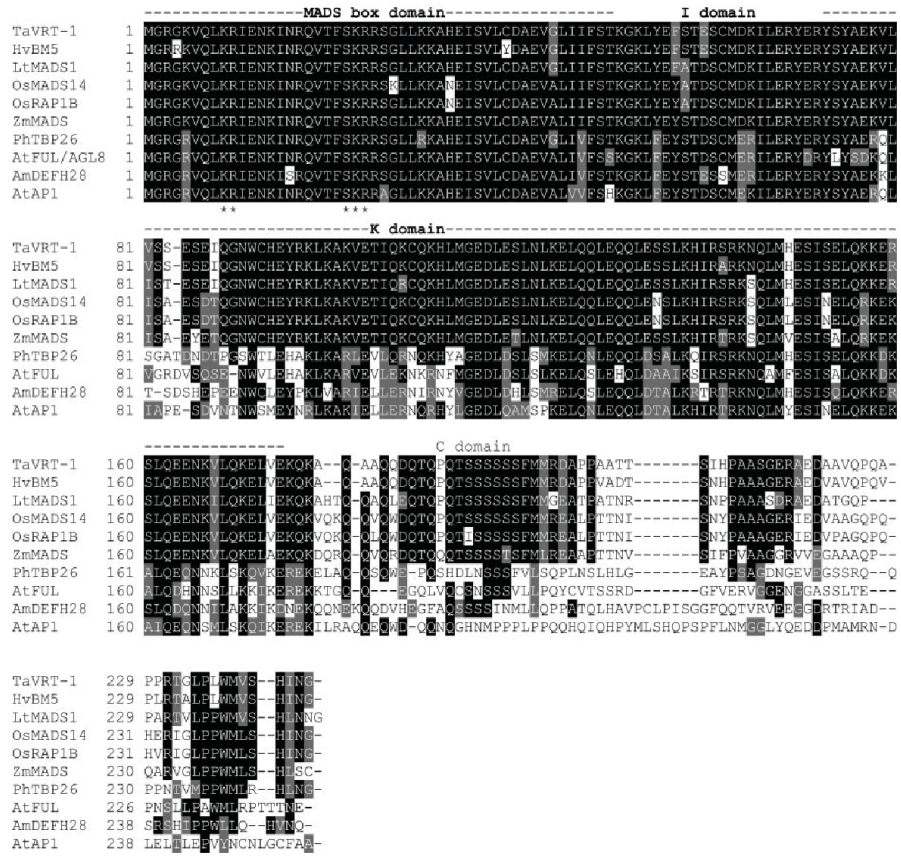

Figure 4. Sequence alignment of members of AP1/SQUA family. Wheat (*Triticum aestivum*) *Ta*VRT-1, Barley (*Hordeum vulgare*) *Hv*BM5, *Lolium temulentum Lt*MADS1, indica rice *Os*MADS14, japonica rice *Os*RAP1B, maize (*Zea mays*) *Zm*MADS, petunia (*Petunia hybrida*) *Ph*TBP26, *Arabidopsis At*FUL/AGL8, *Antirrhinum majus Am*DEFH28, and *Arabidopsis At*AP1. Identical and similar amino acids are shaded, respectively, in black and gray. MADS-box domain, DNA-binding domain; I, intervening region; K, keratin-like domain; C, C-terminal region. (Adapted from Danyluk *et al.*, 2003).

In vernalization requiring-varieties, regulatory interactions between VRN1, VRN2 and VRN3 integrate vernalization and long day responses. Flowering during fall, when days are still long, is prevented by the VRN2-dependent downregulation of *VRN3* and, at this time, *VRN1* is expressed at a very low level, both in leaves and apices. During the cold and short days of winter, *VRN1* is gradually upregulated and downregulates *VRN2*. Low levels of *VRN2* promote the upregulation of *VRN3* in leaves during the long days in spring. Then, VRN3 moves from the leaves to the shoot apex, where it further promotes the *VRN1* transcription above the threshold required for flowering (Trevaskis *et al.*, 2007a; Distelfeld *et al.*, 2009).

In winter varieties, the initial expression level of *VRN1* is low and is induced by vernalization. The extent to which *VRN1* is induced depends on the length of the vernalization exposure, resulting in a quantitative effect of the timing of the inflorescence initiation: the longer the plant is subjected to cold, more the *VRN1* transcript abundance increases, and this parallel the degree to which flowering is accelerated (Danyluk *et al.*, 2003; Murai *et al.*, 2003; Trevaskis *et al.*, 2003; Yan *et al.*,

2003; von Zitzewitz *et al.*, 2005). In spring varieties, *VRN1* expression increases during inflorescence initiation, and remains high during the reproductive stages of the shoot apical meristem development (Trevaskis *et al.*, 2007a). Thus, VRN1 is required for the vernalization response, as well as to establish and maintain the inflorescence meristem identity during shoot apex development (Trevaskis *et al.*, 2007b). The activation of *VRN1* expression in leaves unlocks the long day flowering response, allowing the plants to further accelerate the reproductive development post-vernalization; whereas *VRN1* activation in shoot apices promotes the transition to reproductive development (Hemming *et al.*, 2008; Preston and Kellogg, 2008; Sasani *et al.*, 2009). Furthermore, deletion of *VRN1* and flanking genes do not allow plants to flower (Shitsukawa *et al.*, 2007; Distelfeld and Dubcovsky, 2010).

The difference in *VRN1* expression between barley winter and spring varieties is linked to the presence or the absence of the first intron, or a deletion in the first intron. The first intron of *VRN1* contains a region required to maintain the gene in a repressive state prior to winter (Fu *et al.*, 2005; von Zitzewitz *et al.*, 2005; Cockram *et al.*, 2007; Szücs *et al.*, 2007; Hemming *et al.*, 2009). Moreover, alleles lacking a large part of the first intron are actively expressed, and are associated with an early vernalization-independent flowering (Szücs *et al.*, 2007; Hemming *et al.*, 2009). The state of the chromatin at the *VRN1* locus is very important to determine its activity. Without vernalization, the *VRN1* locus shows high levels of histone 3 lysine 27 tri-methylation (H3K27Me3), which is a repressive histone modification typically associated with an inactive chromatin state (Oliver *et al.*, 2009). This modification, found within the *VRN1* first intron, might contribute to its repression prior to winter.

2.2.2. The gibberellin pathway in cereals

The ability of GA to induce bolting and flowering was first described in 1957 in different plant species (Lang, 1957), and this function is conserved in grasses. Exogenous applications of GA are sufficient to accelerate flowering time in vernalized *Lolium perenne* (MacMillan *et al.*, 2005), and in wheat spring varieties grown under long days, as well as in vernalized winter varieties (Evans *et al.*, 1995). The upregulation of VRN3 in leaves, and its transport to the wheat shoot apical meristem, are sufficient for the induction of *VRN1* in the shoot, and for the induction of the genes involved in GA biosynthesis, both required for a correct spike development in wheat (Pearce *et al.*, 2013). Moreover, the requirement of GA and the regulation of GA biosynthesis genes FT-dependent ensure that the floral meristem does not completely develop during the long spring days, protecting the developing spikes from potential low temperature damage (Pearce *et al.*, 2013).

2.2.3. The *Sdw1/Denso* gene locus in cereals

In cereal crops, plant height determines the overall plant architecture, and it is also closely associated with grain yield. The semi-dwarf trait is very desiderable because of reduced lodging, and the potential of increase the grain yield, as happens in semi-dwarf rice, barley and wheat cultivars (Xu *et al.*, 2017).

The barley semi-dwarfness is caused by a deficiency in the gibberellin synthesis pathway, and short-stature barley plants are known to be GA-sensitive and respond to applied GA (Kuczyńska *et al.*, 2013). Modifications in the gibberellin pathway have been advantageous in crop breeding, conferring the semi-dwarf phenotype. An example is the rice semi-dwarf mutant *sd1*, where the reduced function of a GA20-oxidase-2 enzyme causes a reduction in plant height (Jia *et al.*, 2009, 2011, 2015).
The GA biosynthesis pathway depends on the function of several enzymes, and the *GA20ox*, *GA3ox*, and *GA2ox* encode enzymes required for the homeostasis of the endogenous amount of gibberellin in plants (Olszewski *et al.*, 2002). Moreover, there is an association between these genes and the quantity of GA. Low GA concentration induces the expression of genes which promote GA biosynthesis, while increased GA expression represses *GA20ox* and *GA3ox* genes, decreasing the GA quantity. Conversely, the expression of *GA2ox* genes is stimulated by elevated GA concentration, producing inactive GA (Hedden and Phillips, 2000; Olszewski *et al.*, 2002). Impairment in the mode of operation of any of these genes can affect plant height. Loss of *GA20ox* or *GA3ox* functions cause a decrease in the endogenous GA level, and leads to reductions in plant height; whereas their overexpression stimulates an extensive growth. Conversely, an enhanced expression of *GA2ox* causes a dwarf phenotype, while a loss of its function intensifies an elongation of the internodes (Jia *et al.*, 2009, 2011, 2015; Kuczyńska *et al.*, 2013, Xu *et al.*, 2017).

The *Sdw1*/*Denso* locus in barley controls plant height, and it also influences several agronomic and quality traits (Jia *et al.*, 2011). The main phenotypic effect of *sdw1*/*denso* is a 10-20 cm reduction of plant height, but this gene also cosegregates with several QTLs controlling different traits in barley, such as yield, height, and heading date. Semi-dwarf barley plants have an increased heading date, late maturity, and decreased grain weight (Jia *et al.*, 2009, 2011, 2015; Kuczyńska *et al.*, 2013). Comparative mapping revealed that the S*dw1*/*Denso* region in barley is syntenic to rice *Sd1* gene located on the chromosome 1, which encodes a GA-20 oxidase enzyme. The gene isolated in barley shows a conserved gene structure, three exons and two introns, and a high degree of sequence similarity with *Sd1*, 88.3% genomic sequence similarity and 89% amino acid sequence identity (Jia *et al.*, 2009). Both, *sdw1*/*denso* from barley and *sd1* from rice are sensitive to exogenous applications of GA_3. Moreover, the *HvGA20ox2* expression levels in barley

correlate with plant height and its expression pattern is similar to *Sd1*. Therefore, it is possible to conclude that *Sdw1*/*Denso* gene in barley is an orthologue of *Sd1* (Jia *et al.*, 2009; Xu *et al.*, 2017).

3. Cold acclimation and freezing tolerance

Plants show a variety of responses to the surrounding environment, and some of these responses require the accumulation of information on temperature for weeks, such as the vernalization-induced flowering. Ultimately, extreme environmental conditions challenge plant survival and the ability to adjust the flowering process plays an important role in the adaptation to different environments.

3.1. In *Arabidopsis thaliana*

The cold acclimation process, defined as the gain of tolerance to freezing temperature after to be subjected to a short period of non-freezing temperatures (Thomashow *et al.*, 1999), has been well characterized in *Arabidopsis*. Environmental temperature strongly affects plant growth and development, and plants use endogenous hormones to connect the growth rate with temperature. In particular, gibberellin signaling has a central role in regulating many aspects of plant development and, especially, in the control of floral transition in a wide range of different species. In *Arabidopsis*, GA promotes flowering by acting directly on the expression of *LFY* (Blázquez *et al.*, 1998; Blázquez and Weigel, 2000). The temperature signaling pathway regulates flowering converging on FT. However, there is no evidence for a direct role of GA in temperature-dependent flowering. Variations in temperature can have dramatic effects on flowering time, increasing temperature promotes flowering with a shorter vegetative phase. In *Arabidopsis*, the autonomous pathway regulates flowering by the maintenance of *FLC* downregulation, and mutants in this pathway are insensitive to the promotion of flowering by increasing temperature (Blázquez *et al.*, 2003), this suggests that *FLC* has a determinant role in temperature-regulated flowering. Studies of natural variation in temperature responsive flowering in *Arabidopsis* have shown that the temperature promotion even occurs in vernalized plants, or in plants with constitutively low *FLC* expression. This suggests that, despite the strong epigenetic repression of *FLC*, temperature-dependent variation in FLC activity has a pivotal importance in flowering (Balasubramanian *et al.*, 2006). The ambient growth temperature affects *FLC* transcript levels, and *flc-3* loss-of-function mutants retain temperature responsive flowering (Blázquez *et al.*, 2003; Lee *et al.*, 2007). Therefore, it is likely that FLC functions redundantly in the temperature regulation of flowering, perhaps with FLOWERING LOCUS M (FLM) and SHORT VEGETATIVE PHASE (SVP), both of which also control *FT* transcription and have a role in the temperature response (Lee *et al.*, 2007).

Additionally, in *Arabidopsis*, low temperatures regulate the expression of genes involved in the gibberellin biosynthesis and deactivation pathway. In particular, two *GA20ox* genes are downregulated and one *GA2ox* gene is upregulated; these changes in their expression suggest that in cold the amount of endogenous GA is increased (Lee *et al.*, 2005). The GA accumulation might be part of the vernalization mechanism which promotes flowering, or might be that GA affects cold stress signaling and tolerance (Lee *et al.*, 2005). Moreover, in imbibed seeds, cold treatment activates *GA3ox1*, enhancing the GA biosynthesis and promoting seed germination (Yamauchi *et al.*, 2004).

3.2. In cereal crops and barley

In cereals, plant development is mostly affected by photoperiod and vernalization requirements. The day length influences several developmental processes, such as apical morphogenesis and leaf production (Kirby, 1969). When cereals are grown in short day conditions, the vegetative phase become longer: the number of leaves is increased and the reproductive phase, marked by the appearance of the double ridge structure, is delayed (Mahfoozi *et al.*, 2000, 2001). Long day conditions accelerate floral initiation and heading by reducing the number of leaves. Vernalization accelerates flowering and in vernalization requiring cereals the vegetative phase is shorter by decreasing the number of leaves (Chouard, 1960; Wang *et al.*, 1995).

Barley and wheat winter varieties can adapt to low temperatures and, subsequently, are able to survive the winter. Cold acclimation and vernalization response are interconnected: the longer the plants are exposed to low temperatures, the more the frost tolerance increases. This continues until the vernalization saturation point, when further cold treatment has no additional impact on flowering time and the frost tolerance begins to decrease (Mahafoozi *et al.*, 2001; Prášil *et al.*, 2004). Genetic studies support the idea that in fully vernalized plants, the activation of *VRN1* might be the cause of the decreasing of the frost tolerance. Varieties with active allele of *VRN1* have a significant reduction in their ability to acclimate to cold in comparison to lines with wild type *VRN1* alleles, which require vernalization to flower (Koemel *et al.*, 2004; Limin and Flowler, 2006). Actually, it is not clear if the decrease of the frost tolerance is a direct or an indirect consequence of the *VRN1* activity but, apart from the mechanism, the apparent relationship between *VRN1* activity and frost tolerance has very important implications, since altering the vernalization requirement can also affect winter survival (Trevaskis, 2010).

In wheat *Rht3* dwarf varieties, the Rht3 protein fails to interact with GID1, causing a block in the GA responses and an extreme dwarfism (Wu *et al.*, 2011). These plants grow normally at low temperatures but, differently from wild type, they are not able to increase their growth rate when

the temperature is increased to 20°C (Tonkinson *et al.*, 1997). Moreover, applied GA promotes wheat growth in low temperatures, suggesting that the amount of bioactive GA in these plants is subjected to temperature regulation (Tonkinson *et al.*, 1997).

The thermoperiodic growth response, defined as promotion of growth by increasing the difference between day maximum and night minimum temperature (Went, 1944), give more detailed evidence of the role of gibberellin in temperature responses. At the molecular level, the stem thermoperiodic growth in pea is correlated with the transcriptional repression of *GA2ox* genes, and with the increase in the transcript levels of the genes required for the biosynthesis of gibberellin (Grindal *et al.*, 1998; Stavang *et al.*, 2005). Moreover, in citrus the increase in temperature is linked to an increase in the transcript of *GA20ox* genes and, therefore, to an increased amount of bioactive GA (Vidal *et al.*, 2003).

4. Shoot apical meristem development in barley

The study of the morphological development of barley spike has big importance for the agronomist. It allows determining the period during which spike development can be affected by the environment, it can provide information about variation in the mature spike analyzing how the early spike develops, and it allows the analysis of the effect of the environmental changes on the spike development (Bonnet, 1935). The Waddington scale is a very helpful method to characterize the various steps of spike development on the basis of the morphogenesis of the floral parts (Waddington *et al.*, 1983). The different stages, according to the Waddington scale, are described below.

The primary apical meristems of the plant are the site of the post-embryonic organogenesis and are situated at the tip of the shoot and root. The shoot apical meristem is responsible for initiating the above-ground structures during the vegetative development, such as nodes, internodes, leaves, axillary meristems and inflorescence (Sussex, 1989). The mature barley inflorescence is named spike and consist of the floral stem or rachis and floral units called spikelets. Each spikelet is formed by a floret and two bracts, called outer glumes, and each rachis node sustains three spikelets. In the two-row barley cultivars, including the wild barley, only the central spikelet is fertile, while the lateral ones are sterile and do not develop. In the six-row barley cultivars all three florets develop and produce grains (Kirby and Appleyard, 1987; Komatsuda *et al.*, 2007). In spring barley varieties, the transition from the vegetative to reproductive phase occurs under favorable conditions of light and temperature during early stages of seedling development. At the end of the vegetative phase, a stage called transition apex (Waddington stage 1), the stem just begun to elongate in preparation for the spike differentiation and the single ridges are still developed. The

transition of the shoot apical meristem to an inflorescence meristem is marked by the appearance of the double ridge structures, instead of single ridges. The double ridge corresponds to a pair constituted of a leaf primordium and a lateral meristem (Kirby and Appleyard, 1987). The stages named early double ridge and double ridge (Waddington stages 1.5 and 2; Figure 5B) are characterized by the emergence of the first floret primordia and by the specification of the reproductive shoot apical meristem. With the progression of the inflorescence development, leaf initials fail to develop and the lateral meristems become the main growing points. From the lateral meristems will originate the spikelet triplet meristem (STM), which will develop in three spikelet meristem (SM), one central and two lateral (Bossinger *et al.*, 1992), the triple mound stage (Waddington stages 2.25; Figure 5C). From the SM a floret meristem (FM) will arise. At the base of the FM, two outer glume primordia (OGP) originate. The OGPs develop into the outer glumes and from the FMs arise the floral organ primordia. The subsequent stages, glume, lemma, and stamen primordium stages (Waddington stages 2.5, 3, and 3.5; Figure 5D), differentiate into mature spikelet structures, the first floral organ primordia differentiates and the stem elongation initiates (Bossinger *et al.*, 1992). Along the immature inflorescence axis several stages of spikelet development can be distinguished. Differentiation is not synchronous. In the central region, the spikelets develop earlier than the basal and the apical spikelets. At the awn primordium stage, with the carpel extending round three sides of the ovule (Waddington stage 5; Figure 5E), the apex stops to initiate new SMs, the final number of spikelet primordia is defined and the structural layout of spikes and spikelets is established (Kirby and Appleyard, 1987). Next, the spike further develops and differentiates, to be ready for the subsequent fertilization, caryopsis development and grain filling. These processes determine the final number and the final size of the grains produced by each spike (Sreenivasulu and Schnurbusch, 2012). Even the development of central and lateral spikelets occurs in a non synchronous way. The lateral spikelets develop more slower in rudimentary and sterile structures that, together with the developed and fertile central spikelets, form the barley inflorescence known as “two-row spike”, peculiar of the wild and most cultivated barley. The spikelet axis, also called rachilla, bears the outer glumes and the lemma; the lemma is an abaxial floral bract which surrounds a single floret and carries the awn, a bristle-like distal appendage. Together, the lemma and the awn can be considered as a reduced vegetative leaf. (Pozzi *et al.*, 2000). The floret comprises two palea, two lodicules, three stamens and the pistil. Usually barley is an autogamous species, but the lodicules can swell up, pushing apart the palea and the lemma, allowing anther extension and cross-pollination (Nair *et al.*, 2010).

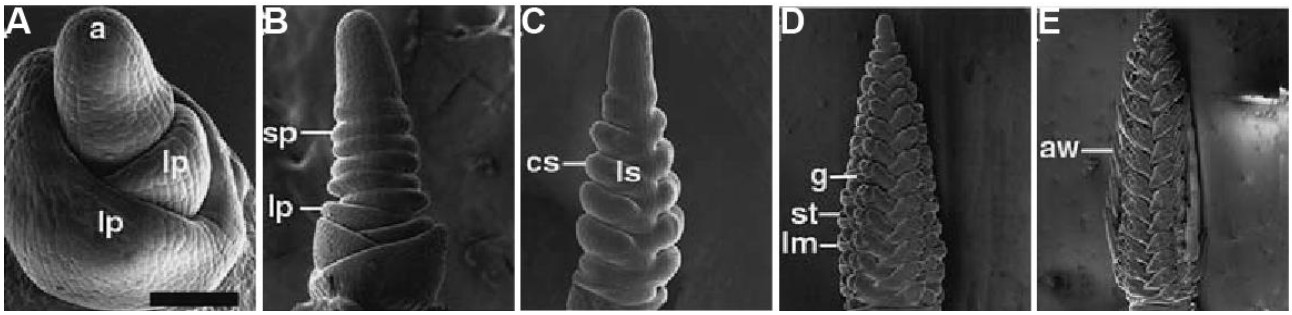

Figure 5. Scanning electron micrographs of wild type barley vegetative and inflorescence shoot apex. (A) Vegetative stage, 8 days old seedlings; (B) double ridge stage, 13 days old seedlings; (C) triple mound stage, 14 days old seedlings; (D) stamen primordium stage, 19 days old seedlings; (E) awn primordium stage, 23 days old seedlings. Scale bar in A equals: A 74 µm; B 174 µm; C 222 µm; D 544 µm; E 1.16 mm. a, apex; aw, awn; cs, central spikelet; g, glume, lm; lemma; lp, leaf primordium; ls, lateral spikelet; sp, spikelet primordium; st, stamen primordium. (Modified from Babb and Muehlbauer, 2003).

AIM OF THE PROJECT

In *Arabidopsis*, cold temperature delays growth and flowering. At the molecular level, this growth restriction can be explained by the stimulated GA catabolism, that results in the accumulation of DELLA proteins, major plant growth and flowering time repressors of the gibberellin signaling pathway (Achard *et al.*, 2008a; Schwechheimer, 2012). This DELLA imposed delay of growth and flowering can be relieved by GA-promoted DELLA degradation.

In our laboratory, it has been shown that the *Arabidopsis* MADS-box transcription factor APETALA1 (AP1) is repressed by direct interactions with DELLAs. Moreover, it has been demonstrated that in *Arabidopsis* plants grown in cold temperature the RGA accumulation fails following GA treatments.

Since DELLA protein levels increase at low temperature and *AP1* overexpression causes early flowering in *Arabidopsis* (Mandel and Yanofsky, 1995), we hypothesize that the increased AP1 activity may render the plants less sensitive to the repressive effects of DELLAs on AP1, to temperature, and to temperature-dependent GA control of flowering. In line with our results that DELLA directly regulate AP1, as well as the fact that GA and DELLA levels are modulated in response to temperature, we found that flowering time in cold temperature can be promoted by GA applications. Taking together, these data suggest that AP1 is a good candidate for to be an important regulator that integrates flowering inductive stimuli and promotes flower development. Moreover, our results could indicate (i) that AP1 has an important role as a flowering time regulator in *Arabidopsis*; (ii) that the proposed mechanism may explain the regulation of flowering time and floral development by GA because AP1 is a regulator of both these processes; (iii) since *VERNALIZATION1* (*VRN1*) is the major flowering promoting loci in barley (Danyluk *et al.*, 2003; Trevaskis *et al.*, 2003; Yan *et al.*, 2003, 2004a), that the AP1-DELLA interaction and regulation can be conserved in monocots and can explain how gibberellin controls flowering in monocots.

Our data have a very relevant meaning because *VRN1*, the barley orthologue of *AP1*, is the major flowering time regulator in barley, and an interaction between VRN1 and SLENDER1 (SLN1), the DELLA counterpart in barley, could explain, at least partially, the gibberellin regulation of flowering in cold temperature in this species. In fact, a VRN1-dependancy of GA responses has been demonstrated (Pearce *et al.*, 2013), and it is in line with the molecular mechanism proposed here.

The aim of this project is the examination of the regulatory mechanism that underlies the control of the flowering time regulation by the phytohormone gibberellin, in the context of cold temperature in barley. I want to examine the differential VRN1- and SLN1-dependent, as well as GA-suppressible effects of cold induced growth inhibition in barley, using selected lines to gain insights into the

physiological relevance of the VRN1-SLN1 interaction in this crop species. Specifically, I want to elucidate how cold temperatures and GA applications can modulate the activity of the flowering time regulator VRN1 from barley both in terms of time to flower and expression levels.

The examination of the VRN1-SLN1 interaction will shed a light on the regulation of flowering time and floral development by VRN1 and gibberellins, and, thereby, elucidate the pleiotropic effects of VRN1, and its interplay with GA in the control of flowering.

In *Arabidopsis*, the genes involved in the biosynthesis and catabolism of gibberellins are subjected to cold and GA regulation (Hedden and Phillips, 2000; Olszewski *et al.*, 2002). Further analysis should highlight how cold and GA modulate the expression levels of the GA biosynthesis genes in barley, giving me the chance to understand better how this biosynthetic pathway is regulated in cereal species.

Additionally, I want to test if the AP1-DELLA interaction identified in *Arabidopsis* also takes place between VRN1 and SLN1 in barley.

RESULTS

1. Effect of low temperature and gibberellin on flowering time in barley

In barley, *VRN1* is the major flowering time regulator with a pivotal role in the control of the transition of the shoot apex from vegetative to reproductive development (Danyluk *et al.*, 2003; Trevaskis *et al.*, 2003; Yan *et al.*, 2003, 2004a). In wheat, plants carrying a deletion in the *VRN1* gene never flower (Shitsukawa *et al.*, 2007) and a proper spike development requires the simultaneous presence of gibberellins and VRN1 (Pearce *et al.*, 2013). My interest was to elucidate if high VRN1 levels cause early flowering in barley plants, if the high VRN1 level can overcome the delay in growth and flowering due to DELLA accumulation in cold temperatures, and if gibberellin treatments can affect flowering in barley.

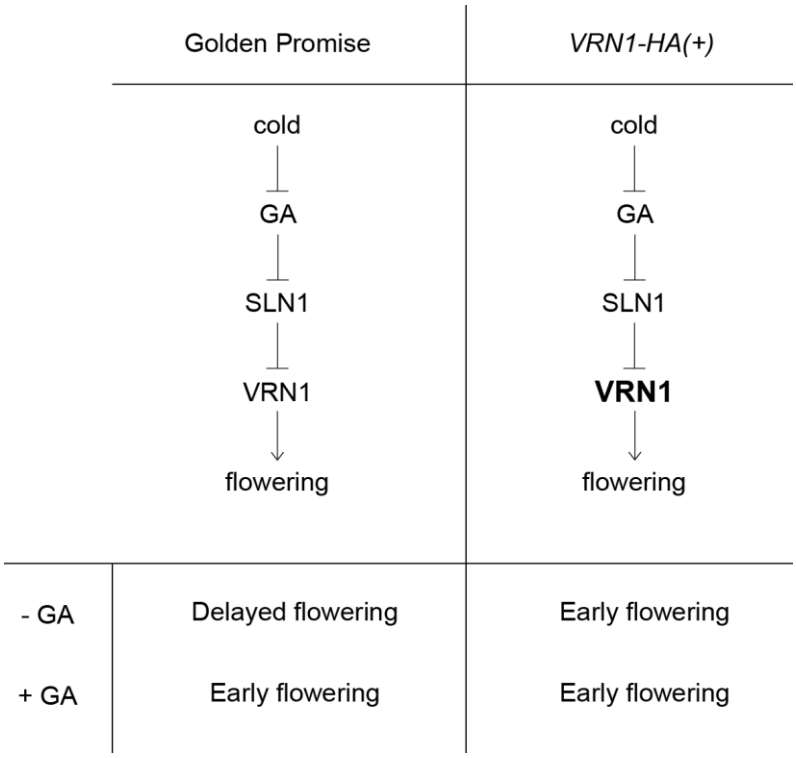

Figure 6. Scheme representing the cold imposed growth inhibition in barley based on a hypothetical VRN1-DELLA antagonism. In cold temperature, Golden Promise is late flowering and I hypothesize that GA applications can rescue its late flowering phenotype; whereas, transgenic barley plants *VRN1-HA(+)* in cold temperature should show an early flowering phenotype, regardless of GA treatment.

For this purpose, I set up an experiment (Figure 6), using Golden Promise, a spring barley variety without vernalization requirement and transgenic barley plants expressing *VRN1* in the Golden Promise background [*VRN1-HA(+)*]. The *VRN1-HA* transgene construct carries the *VRN1* genomic

sequence fused to six copies of the HA-tag (YPYPDVPDYA) driven by the *VRN1* endogenous promoter. It has the 3' UTR of VRN1, but a large part of the first intron is missing. This transgene leads to the plants having a high basal expression level of *VRN1* in comparison to Golden Promise plants (Deng *et al.*, 2015).

Additionally to Golden Promise and *VRN1-HA(+)* plants, I used Himalaya and *grd5* (M574) plants. These plants carry an active *Ppd-H1* allele and, consequently, they are sensitive to photoperiod. *Ppd-H1* is the major regulator of photoperiod response in barley and *ppd-h1* mutants show a late flowering phenotype, and a reduced response to photoperiod, due to an altered expression of the photoperiod pathway genes *HvCO1* and *HvCO2*, and a consequent reduced expression of their downstream target *HvFT1* (*VRN3*), a key regulator of flowering (Andrés and Coupland, 2012; Turner *et al.*, 2005). *grd5* (M574) plants carry a frameshift mutation, resulting in an early stop codon, in *Grd5* (*GIBBERELLIN-RESPONSIVE DWARF5*) gene, encoding the *ent*-kaurenoic acid oxidase enzyme, which catalyzes the oxidation of the *ent*-kaurenoic acid for the production of GA_{12} (Helliwell *et al.*, 2001). M574 is a weak allele of *grd5*, in which the developing grains accumulate *ent*-kaurenoic acid, but GA_1 is still detectable and the presence of GA_{12} might be due to a residual activity of *Grd5*. This mutant shows a gibberellin-responsive dwarf phenotype (Helliwell *et al.*, 2001), and for this reason was included in the experiment. It worked as an internal control of the experiment, to be sure that the gibberellin treatment was working.

1.1. Low temperature and gibberellin affect flowering time in barley

In order to evaluate if cold and applied gibberellin could affect the flowering time in barley, I performed two different experiments. In the first one, I grew barley plants at 15°C, in order to have a quantification of the heading date (flowering time) in conditions that are normal for barley. In the second, I grew barley in cold conditions, at 8°C, to evaluate the effect of low temperatures on the heading date. Moreover, I treated some of these plans with GA_3, to understand the involvement of gibberellin in the control of this process. Both experiments were performed in long day conditions (16 hours light/8 hours dark).

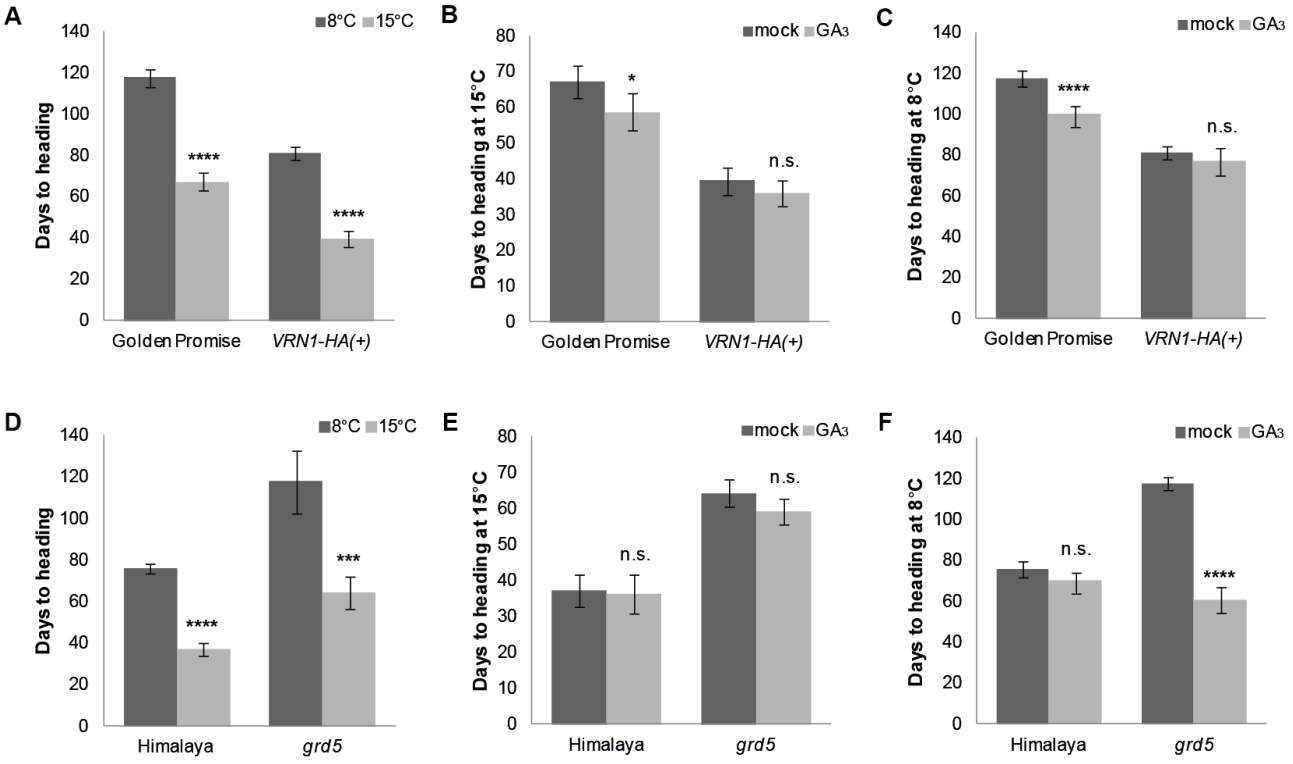

Figure 7. Heading date of barley plants. (A and D) Comparison between plants grown at 8°C and at 15°C; (B and E) mock- and GA_3- treated plants grown at 15°C; (C and F) mock- and GA_3- treated plants grown at 8°C. Asterisks indicate *P*-values of Student's *t*-test. (*P<0.05; ***P<0.001; ****P<0.0001; n.s., not significant).

Cold temperatures caused a very strong and significant delay in flowering time in all genotypes analyzed (Figure 7A and 7D). In Golden Promise, low temperatures delayed flowering by 50.2 days, this delay was only 41.8 days for *VRN1-HA(+)* transgenic lines. Further, the transgenic lines flowered earlier than Golden Promise in cold as well as in normal growth temperature conditions. In fact, at 15°C the difference in the heading date between Golden Promise and *VRN1-HA(+)* plants is 36.2 days, at 8°C the difference is 27.8 days (Figure 7A). These data confirm that a high basal expression level of *VRN1* has a strong effect in promoting flowering, and that in low temperatures the high VRN1 level can overcome the repressive effect of cold on flowering time.

Himalaya plants grown in low temperatures and under inductive photoperiod (long days, 16 hours light/8 hours dark), delayed their flowering by 39 days in comparison to normal growth conditions (Figure 7D). They flowered earlier than Golden Promise, at 15°C the difference in the heading date is 30.2 days and at 8°C is 41.5 days, highlighting the strong impact of the presence of an active *Ppd-H1* allele on flowering time control. As already mentioned, *grd5* mutants are impaired in the gibberellin biosynthesis pathway and, in comparison to Himalaya plants, they showed a late flowering phenotype in both temperature conditions (Figure 7D). At 15°C the difference in the

heading date between *grd5* and Himalaya is 27.33 days and at 8°C is 41.7 days. This confirms that a functional GA signaling pathway is required for a proper flowering.

Exogenous application of gibberellin in normal growth conditions, 15°C, seemed to have a weak or no effect on the heading date of the plants analyzed (Figures 7A and 7D).
In GA_3-treated Golden Promise plants, the heading date was promoted by 10 days (Figure 7B), suggesting a role of GA in flowering time control. However, GA treatments did not have any effect in promoting flowering in *VRN1-HA(+)* transgenic lines (Figure 7B).

Applied GA did not have any effect on the heading date of Himalaya plants (Figure 7E). The lack of difference in the heading date between mock- and GA_3- treated Himalaya plants could be linked to the active photoperiod response of this cultivar. The *grd5* mutants are responsive to applied GA (Helliwell *et al.*, 2001) and, in normal growth temperature conditions (15°C), the general overall plant development was accelerated upon GA applications (data not shown), confirming that the GA treatment worked. However, the heading date was not significantly affected by the treatment (Figure 7E). These mutants are in Himalaya background then, like the wild type, the photoperiod has such a strong impact on flowering time control, that exogenous applications of GA did not have any further effect in accelerating this process.

When grown in low temperature and treated with GA, Golden Promise heading date was 17 days earlier than in mock-treated plants (Figure 7C). The *VRN1-HA(+)* transgenic lines, even in low temperatures, flowered earlier than Golden Promise, but they were not sensitive to applied GA, the difference in the heading date was not significant (Figure 7C). This implies that, even in cold, the amount of endogenous *VRN1* in this line was enough to accelerate flowering and, moreover, was enough to overcome the hypothesized effect of DELLA accumulation, and application of GA, which stimulates DELLA degradation, had no further effect on *VRN1* when overexpressed.

In low temperatures, mock-treated Himalaya plants showed an early flowering phenotype in comparison to Golden Promise, emphasizing the key role of the photoperiod pathway in promoting flowering. However, these plants seemed to be not sensitive to applied GA, in fact there was no difference in the time to head in Himalaya plants grown in cold temperatures and treated with GA (Figure 7F). Conversely, the *grd5* mutant plants showed a strong response to applied GA in low temperature (Figure 7F). The flowering was anticipated by 56.1 days, and the heading date of GA_3-treated *grd5* mutant plants was very similar to that of Himalaya, suggesting that photoperiod and gibberellin are both required for early flowering in *grd5*.

In conclusion, barley plants showed a late flowering phenotype at 8°C compared to plants grown at 15°C. Moreover, exogenous GA applications could reduce the delay in flowering and growth, especially with a stronger effect promoting flowering time in colder temperature than in warmer temperatures. I could highlight the role of GA and of an active *Ppd-H1* allele in flowering time regulation, and confirm the pivotal role of VRN1 in controlling the transition from vegetative to reproductive phase.

In Golden Promise, exogenous GA promoted flowering, with a stronger effect in low temperatures, suggesting an active role of the gibberellin pathway controlling this process, and that the exogenous GAs compensate for the cold-dependent delay in flowering and growth and for the inactive photoperiod pathway. Moreover, *VRN1-HA(+)* transgenic lines showed an early flowering in comparison to Golden Promise, due to a higher endogenous amount of VRN1. Additionally, these plants did not respond to GA treatment and the elevated amount of VRN1 is sufficient to overcome the repressive effect DELLA accumulation on flowering.

The early flowering phenotype of Himalaya plants, in comparison to Golden Promise, correlates with an active photoperiod response in these plants. Moreover, the non-responsiveness of Himalaya to exogenous GA suggests that the simultaneous presence of an active *Ppd-H1* allele and a functional GA signaling pathway is required to accelerate flowering. Additionally, the *grd5* mutants, impaired in the GA biosynthesis pathway, were late flowering and did not respond to GA treatment 15°C in comparison to Himalaya. However, in low temperature growth conditions they showed an active response to exogenous GA, accelerating their flowering time. These data suggest the requirement of an active GA pathway in promoting flowering, especially in low temperature.

1.2. *Sdw1*/*Denso* gene is required for a proper flowering time in barley doubled haploid lines

The *Sdw1*/*Denso* gene encodes a *HvGA20ox2* enzyme, which is required for the biosynthesis of gibberellin in barley, and its mutation causes a dwarf phenotype and a delay in the heading date (Jia *et al.*, 2009, 2011, 2015; Kuczyńska *et al.*, 2013, Xu *et al.*, 2017).
To investigate how this gene affects the heading date in low temperature growth conditions, and its possible interplay with *VRN1*, I grew four different doubled haploid lines carrying different allele combinations of *VRN1* and *Denso*. The chosen lines came from a doubled haploid population derived from the cross between the Spanish landrace SBCC145 and the German variety Beatrix (Ponce-Molina *et al.*, 2012). These two lines differ for some traits: the SBCC145 is a six-row variety, with low vernalization requirement, early heading, moderate height, and good grain size

(Ponce-Molina *et al.*, 2012). The German Beatrix is a two-row variety, with short stature, high yielding, and has a spring habit (Ponce-Molina *et al.*, 2012). I chose the doubled haploid lines on the basis of the plant height and of the source of *VRN1*. Plants possessing the *sdw1*/*denso* mutation allele are characterized by short stature, and delayed flowering is due to the reduction in the endogenous GA amount (Jia *et al.*, 2005), whereas plants with its wild type allele are characterized by moderate/tall stature and a functional GA signaling pathway. The *VRN1* allele from Beatrix provides a spring growth habit, whereas the *VRN1* allele from SBCC145 requires vernalization (Ponce-Molina *et al.*, 2012). Moreover, the SBCC145 variety, with a low vernalization requirement, carries a *VRN1-4* allele, with a 4079 bp deletion in the first intron, leading to the expression of *VRN1* without vernalization; and, in cold temperatures, *VRN1* expression levels are higher than in normal temperature growth conditions (Hemming *et al.*, 2009).

A

	***Swd1*/*Denso* locus**	***VRN1***	***VRN3***
DH-15	mutant (low GA content)	spring (Beatrix)	late (Beatrix)
DH-120	wild type	spring (Beatrix)	early (SBCC145)
DH-201	wild type	low vernalization requirement (SBCC145)	early (SBCC145)
DH-463	mutant (low GA content)	low vernalization requirement (SBCC145)	early (SBCC145)

B

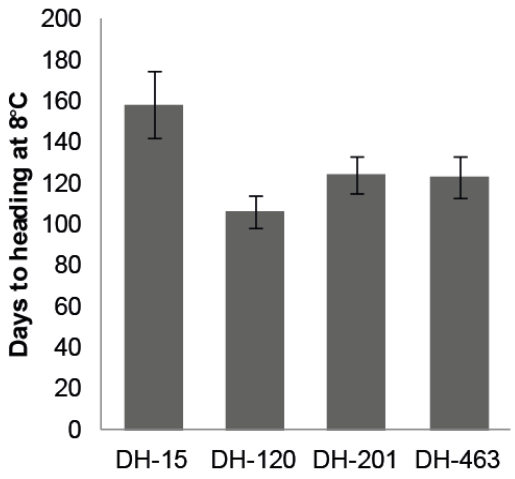

Figure 8. Heading date of barley doubled haploid lines grown at 8°C. (A) Table summarizing the doubled haploid line genotypes, referring to the genes used for the analysis of the heading date. (B) Graph illustrating the heading date of the doubled haploid lines used in the experiment.

The doubled haploid lines DH-15 and DH-120 are spring lines, they inherited the *VRN1* allele from Beatrix. The DH-15 line carries a mutant *sdw1*/*denso* allele, leading to a short stature and a low endogenous amount of GA. The DH-120 line carries a wild type *Sdw1*/*Denso* allele, leading to a tall stature and a high endogenous amount of GA. These two doubled haploid lines showed a difference in the time to flower. The difference in the heading date was 52.5 days, with the DH-15 line showing a late flowering phenotype in comparison to DH-120 (Figure 8). Since it is known that a mutation in the *Sdw1*/*Denso* gene is responsible for a late heading date, it is likely that the later flowering time of the DH-15 line was due to the *sdw1*/*denso* mutation. This further highlights the importance of having a functional GA signaling pathway in order to have a proper flowering time.

The presence of a wild type or a mutated *Sdw1*/*Denso* allele did not seem to have an effect on flowering time in barley varieties with low vernalization requirement, as in the case of DH-201 and DH-463 lines. These two doubled haploid lines differ in the *Denso* allele, wild type in DH-201 and mutated in DH-463, but their flowering time was the same (Figure 8). It is possible that the expression level of *VRN1* was high enough to lead the plants to flower without vernalization, and to overcome the delay in flowering time due to the presence of a mutation in the *Sdw1*/*Denso* gene. Nevertheless, DH-201 and DH-463 lines flowered earlier than DH-15 and later than DH-120 (Figure 8), which are spring varieties and should have higher *VRN1* expression levels.

In consequence, it was necessary to have a look to other genes involved in the control of flowering time. The *VRN3* allele coming from the SBCC145 parental line causes an early flowering phenotype (Ponce-Molina *et al.*, 2012), and it was carried by DH-120, DH-201, and DH-463, whereas the DH-15 line carried the Beatrix allele of *VRN3*. This could be the reason of the later flowering time phenotype of DH-15 in comparison to DH-120. Moreover, DH-201 and DH-463 did not show differences in the heading date (Figure 8), but both inherited the *VRN3* allele from SBCC145. This could explain the same flowering time, even if they carried a wild type *Sdw1*/*Denso* allele, and a mutated *sdw1*/*denso* allele, respectively. Additionally, the early flowering time phenotype given by the *VRN3* allele from SBCC145 would explain the delay in the heading date shown by DH-15 in comparison to DH-120, and the heading date of DH-15 is further delayed by the presence of the mutated *sdw1*/*denso* allele. Furthermore, it could explain the same flowering time showed by DH-201 and DH-463, and their difference in the heading date, in comparison to DH-15 and DH-120. Taken together, these data suggest that the presence of the *VRN3* allele from SBCC145 could overcome the *denso* mutation, which would cause a delay in the heading date. It also explains why the same flowering time was found in DH201 and DH463. Moreover, the combination spring variety, wild type *Sdw1*/*Denso* allele and *VRN3* allele from SBCC145 seems to confer an extremely early flowering time phenotype, as shown in the DH-120 line.

Taken together, these results showed the importance of the GA signaling pathway in regulating flowering time in cold temperature growth conditions. In fact, barley spring varieties carrying a mutant *sdw1*/*denso* allele flowered later than wild type spring varieties with a wild type *Sdw1*/*Denso* allele. Further, these results highlighted the fact that a *VRN3* allele conferring an early flowering phenotype can overcome the delay in flowering caused by a mutation in the *Sdw1*/*Denso* allele.

2. Effect of low temperature and gibberellin on shoot apical meristem development

One week old, mock- and GA_3- treated seedlings did not show any growth differences (Figure 9). However, I could show that the flowering time was affected by temperature and gibberellin treatments. Therefore, I wanted to understand if meristem growth and development were affected in different temperatures and upon GA_3 treatments. For this purpose, I performed two different experiments, growing barley seedlings at 15°C and at 8°C and treating them with GA_3. The samples were collected 8, 9, 10 and 11 days after germination, the meristems were dissected and fixed, and subsequently observed using Nomarski microscopy. In order to determine the developmental stage of meristem development I based my analysis on the Waddington scale (Waddington *et al.*, 1983), and I focused my attention on the emergence of the double ridge structure, which is the morphological marker for the transition from vegetative to reproductive stage in cereals. The appearance of this structure and its development correspond to the Waddington stages 1.5 and 2, which are characterized by the emergence of the first floret primordia, and by the specification of the reproductive shoot apical meristem (Waddington *et al.*, 1983).

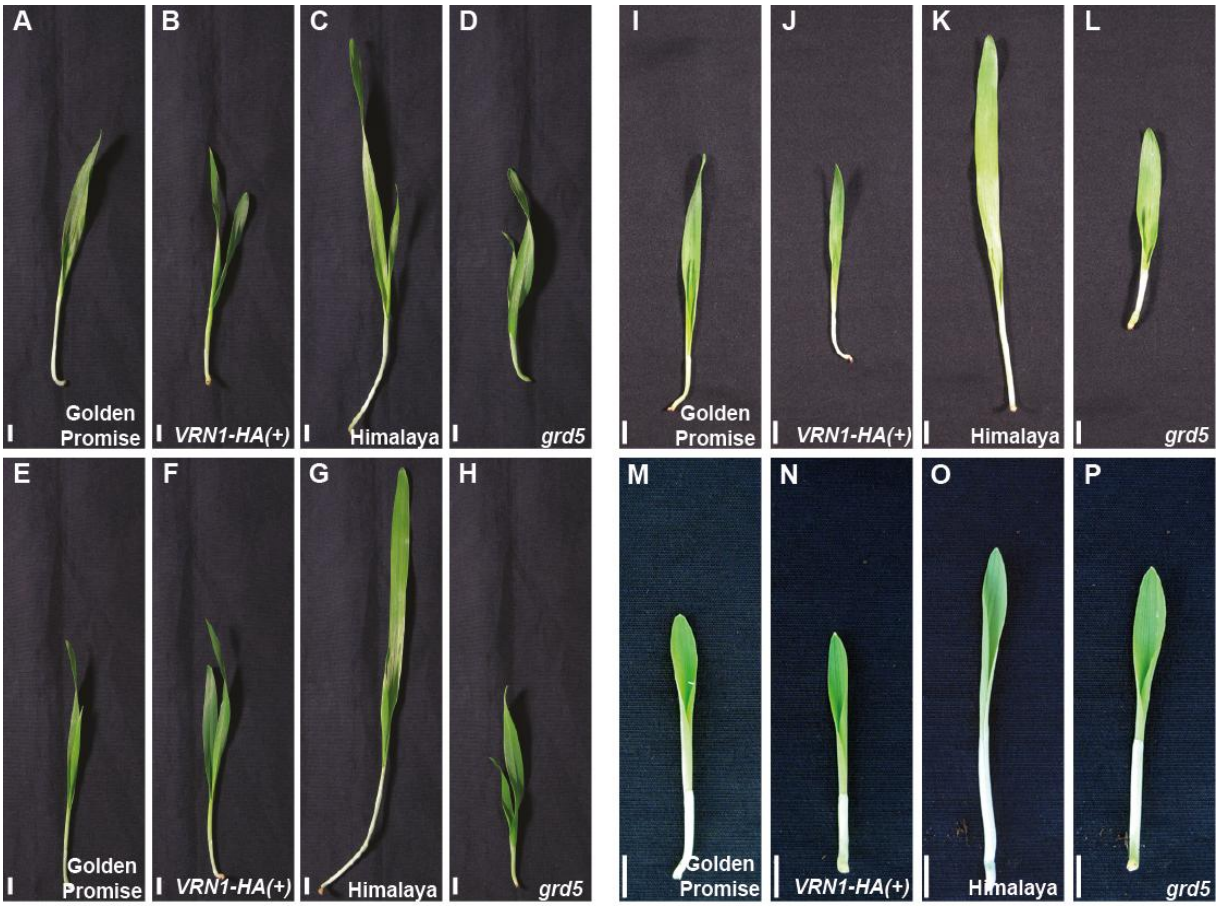

Figure 9. Photographs of one week old barley seedlings grown at 15°C and 8°C. (A-D) Mock-treated seedlings grown at 15°C; (E-H) GA_3-treated seedlings grown at 15°C; (I-L) mock-treated seedlings grown at 8°C; (M-P) GA_3-treated seedlings grown at 8°C. Scale bar 1 cm.

2.1. Meristem development in barley is affected by low temperature and gibberellin

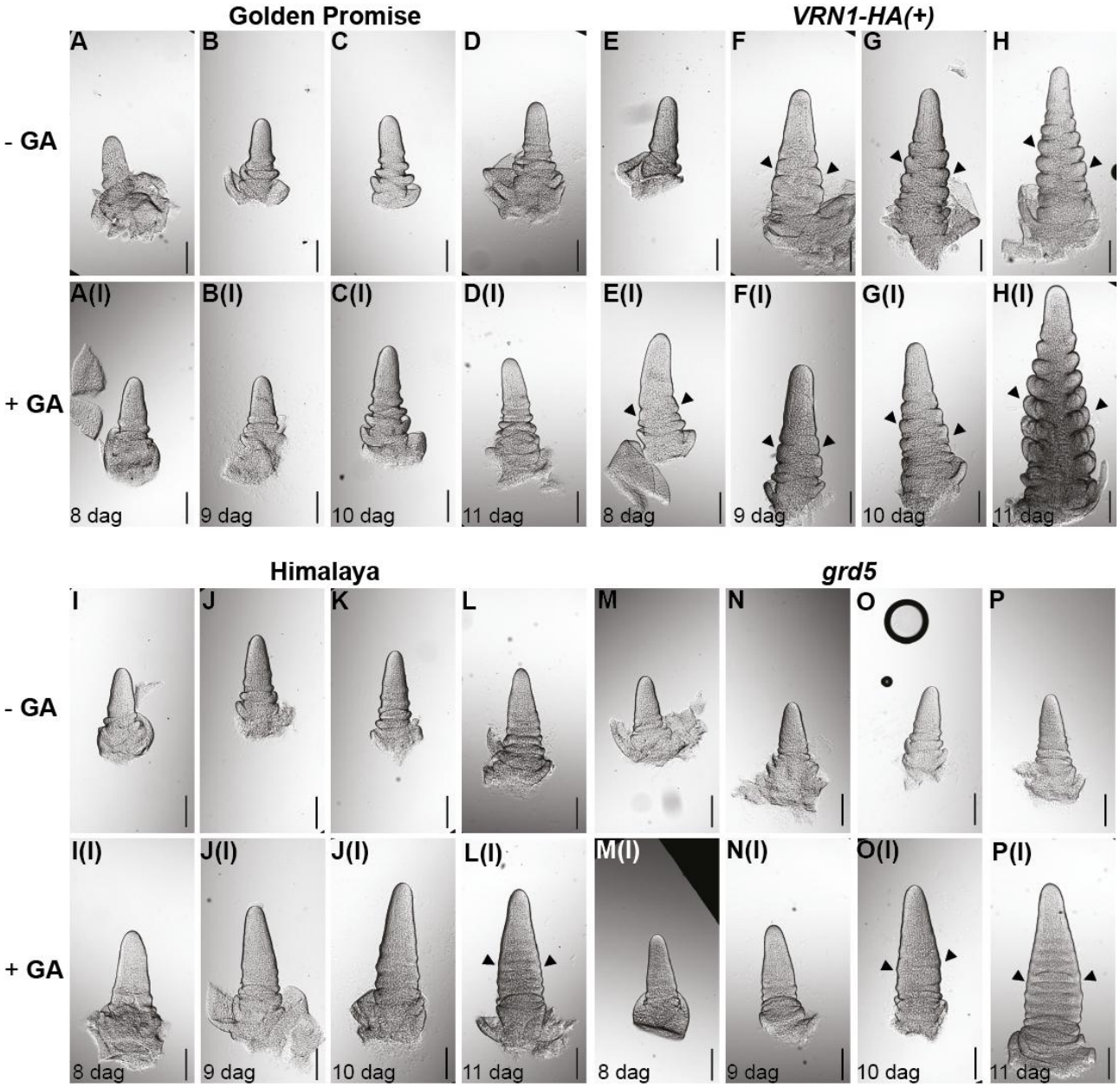

Figure 10. Shoot apical meristems of barley seedlings grown at 15°C. (A-D) Golden Promise treated with mock; [A(I)-D(I)] Golden Promise treated with GA_3; (E-H) *VRN1-HA(+)* treated with mock; [E(I)-H(I)] *VRN1-HA(+)* treated with GA_3; (I-L) Himalaya treated with mock; [I(I)-L(I)] Himalaya treated with GA_3; (M-P) *grd5* treated with mock; [M(I)-P(I)] *grd5* treated with GA_3. Scale bar 200 µm. Arrowheads indicate the early double ridge and the double ridge structure. (*n*=5).

The meristem development of mock-treated Golden Promise seedlings grown at 15°C was delayed in comparison to mock-treated *VRN1-HA(+)* seedlings (Figures 10A-D and 10E-F). This correlated to the higher *VRN1* expression level in the transgenic lines. At 11 days after germination, Golden Promise meristems were close to reach the early double ridge stage (Waddington stage 1.5,

Figure 10D). This means that they were still in the vegetative phase, since the appearance of the double ridge structure is the morphological marker for the transition from vegetative to reproductive phase. Conversely, the *VRN1-HA(+)* lines, at the same time point, already reached the triple mound stage (Waddington stage 2.25), characterized by the presence of spikelet triple meristems (STMs) originating from the lateral meristems (Figure 10H). These results are consistent with the data obtained with the flowering time experiment, which highlighted the fact that the transgenic lines flower earlier than the wild type plants. In conclusions the faster heading date of the *VRN1-HA(+)* lines correlates with the faster meristem development, which is linked to a high amount of *VRN1* in the plant.

In comparison to Golden Promise, Himalaya seedlings grown at 15°C showed a faster meristem development, which was visible only 11 days after germination; even if in both cultivars the meristems were still in the vegetative phase but close to reach the early double ridge stage (Waddington stage 1.5, Figures 10I-L). Since Himalaya plants flowered earlier that Golden Promise, it was likely to suppose that meristem development was accelerated later in time in Himalaya. In *grd5* mutant seedlings, meristem development was slower than Himalaya and it was comparable to Golden Promise. This is in line with the flowering time experiment, which showed that *grd5* mutant plants and Golden Promise had a similar heading date. Moreover, *grd5* seedlings developed the meristem later in comparison to Himalaya and, in a similar manner, the mutant plants flowered later than Himalaya (Figures 10M-P).

Treatments with gibberellin slightly accelerated meristem development in Golden Promise at 10 and 11 days after germination [Figures 10A(I)-D(I)]. The *VRN1-HA(+)* seedlings seemed to be more sensitive to applied GA than Golden Promise. In the transgenic line the triple mound stage was already reached at 10 days after germination [Waddington stage 2.25, Figure 10E(I)], and one day later the meristems initiate the differentiation of the mature spikelet structures, a phase called glume primordium stage [Waddington stage 2.5, Figures F(I)]. These results reflect the flowering time data only for Golden Promise, which showed that the heading date is slightly, but significantly faster at 15°C when exogenous GA is applied. In *VRN1-HA(+)* lines it seems that only the meristems are sensitive to applied GA, because the heading date was not affected by the treatment.

In normal temperature growth conditions, Himalaya as well as *grd5* plants seem to be insensitive to exogenous GA applications with regard to flowering time. Nevertheless, applied GA seemed to have a strong effect on shoot apical meristem development in Himalaya and *grd5* plants [Figures 10I(I)-L(I) and 10M(I)-P(I)]. Already at 8 days after germination, it was possible to observe a significant difference in the size between mock- and GA_3- treated meristems. However, an

acceleration in meristem development was visible at 11 days after germination for Himalaya [Figures 10I(I)-L(I)]. and at 10 days after germination for *grd5*, when the double ridge structure became visible [Waddington stage 2, Figures 10M(I)-P(I)].

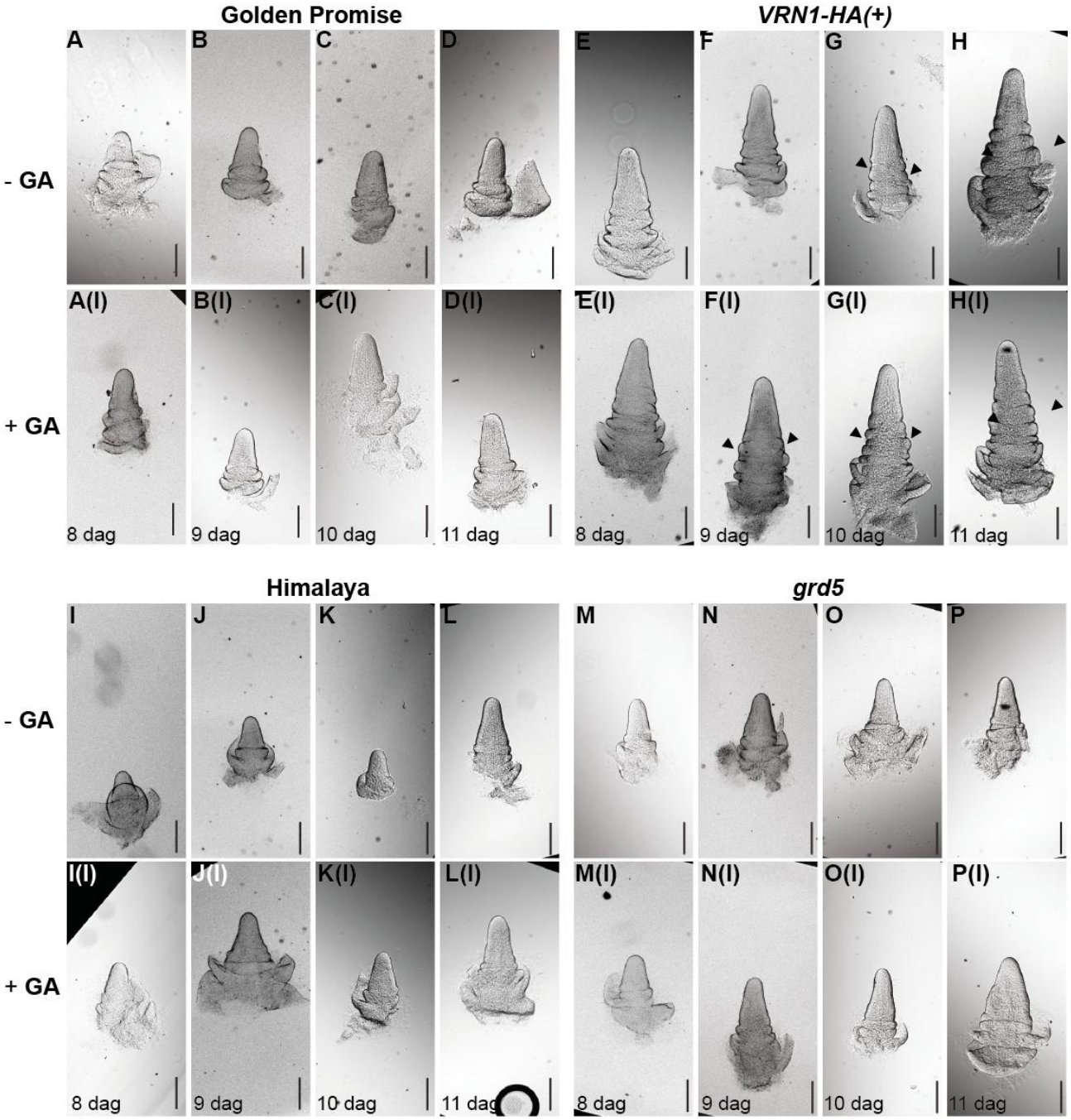

Figure 11. Shoot apical meristems of barley seedlings grown at 8°C. (A-D) Golden Promise treated with mock; [A(I)-D(I)] Golden Promise treated with GA_3; (E-H) *VRN1-HA(+)* treated with mock; [E(I)-H(I)] *VRN1-HA(+)* treated with GA_3; (I-L) Himalaya treated with mock; [I(I)-L(I)] Himalaya treated with GA_3; (M-P) *grd5* treated with mock; [M(I)-P(I)] *grd5* treated with GA_3. Scale bar 200 µm. Arrowheads indicate the early double ridge and the double ridge structure. (*n*=5).

When grown at 8°C, Golden Promise seedlings showed a slower shoot apical meristem development in comparison to 15°C. At 11 days after germination, the meristems of these plants were still in the vegetative phase, and they did not reach yet the double ridge stage (Figures 11A-D). Also the *VRN1-HA(+)* lines delayed their development in low temperature, but they developed faster than Golden Promise. At 11 days after germination, the transgenic lines reached the double ridge stage (Waddington stage 2, Figures 11E-F).

Similarly, in Himalaya as well as in *grd5* seedlings, meristem development was slower in low temperature in comparison to 15°C, and meristems were still in the vegetative phase (Figures 11I-L and 11M-P).

Exogenous applications of gibberellin at 8°C seemed to have no effect on meristem development of Golden Promise seedlings [Figures 11A(I)-D(I)], which did not reflect the faster heading date found after GA_3 treatment, probably due to the fact that the development of fragile plant structures, such as meristems, is delayed in unfavorable conditions. The *VRN1-HA(+)* transgenic lines responded to applied gibberellin, the meristems of GA_3-treated seedlings reached the early double ridge stage one day in advance in comparison to mock-treated seedlings [Waddington stage 1.5, Figures 11E(I)-H(I)]. This result is in line with the data obtained with the flowering time experiment. The transgenic lines did not flower earlier upon GA treatment and their meristem growth did not significantly differ between mock and GA treatments, but their development was still faster than that of Golden Promise lines.

At 8°C, the meristems of Himalaya seedlings did not seem very responsive to exogenous application of GA. Mock- as well as GA_3- treated seedlings meristems were still in the vegetative phase, without any difference between the treatments [Figures 11I(I)-L(I)]. This correlates with the results obtained with the flowering time experiments. The *grd5* mutants seemed to be more sensitive to applied GA than Himalaya, there is a difference in the meristem size between mock- and GA_3- treated seedlings, but the developmental stage was very similar [Figures 11M(I)-L(I)]. This did not reflect the faster flowering of the mutant lines upon GA_3 treatment, and the fact that the overall plant development was very fast in response to applied GA (data not shown).

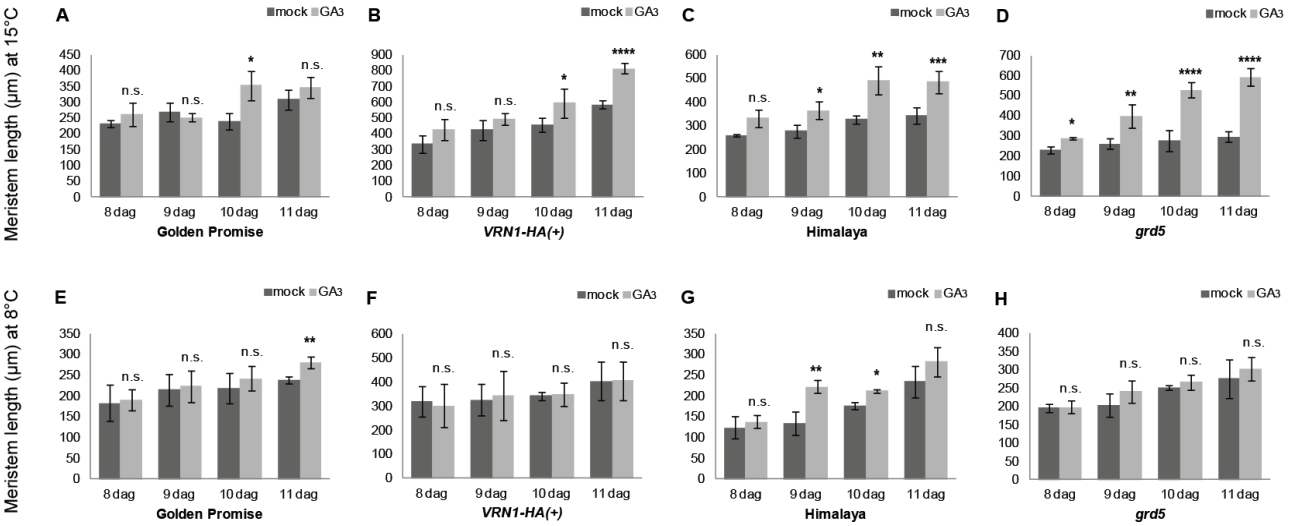

Figure 12. Barley shoot apical meristem lengths. (A-D) Meristem lengths of barley seedlings grown at 15°C; (E-H) Meristem lengths of barley seedlings grown at 15°C. dag, days after germination. Asterisks indicate *P*-values of Student's *t*-test. (*P<0.05; **P<0.01; ***P<0.001; ****P<0.0001; n.s., not significant).

There was only a little correlation between meristem lengths and their developmental stage, when mock- and GA_3- treated seedlings were compared.

In normal temperature growth conditions, Golden Promise showed a significant difference in length at 10 days after germination between mock- and GA_3- treated seedlings; however the developmental stage was very similar (Figure 12A). In *VRN1-HA(+)* transgenic lines, it was possible to observe a difference in length and developmental stage at 10 and 11 days after germination; moreover, at 8 and 9 days after germination, the meristem length was very similar between mock- and GA_3- treated seedlings, but the developmental stage was extremely different (Figure 12B).

In Himalaya, a difference in length mock- and GA_3- treated seedlings was already visible at 8 days after germination, but a difference in the developmental stage became clear at 11 days after germination (Figure 12C). Similarly, in *grd5* mutants, a difference in the developmental stage appeared first at 10 days after germination, whereas the length was significantly different starting from 8 days after germination (Figure 12D).

Even in cold temperature growth conditions, a clear correlation between meristem length and developmental stage did not exist.

In Golden Promise, a significant difference in meristem length was visible at 11 days after germination, but the developmental stage did not change between mock- and GA_3- treated seedlings (Figure 12E). In *VRN1-HA(+)* transgenic lines the meristems did not show any difference in length, but the meristem development was faster upon GA_3 applications (Figures 12F).

Himalaya showed a difference in meristem length at 9 and 10 days after germination, but the meristem development was not accelerated by GA_3 applications (Figure 12G). Similarly, in *grd5* mutant seedlings, meristem length was very similar as well as the developmental stage (Figure 12H).

In low temperature growth conditions (8°C), meristems are smaller in comparison to the ones grew in normal temperature growth conditions (15°C), and it seemed that at 8°C meristems are less sensitive to GA treatments during the days after germination analyzed. This does not exclude that during the next days, meristems can respond in a more significant way to exogenous GA. Moreover, there is a lack of a clear and significant correlation between meristem length and developmental stage, a longer meristem does not mean a progress in development. These data suggest that, since shoot apical meristems are the key structure for seeds propagation, their development is finely regulated in order to ensure the complete maturity until environmental conditions are suitable to have viable seeds.

3. Effect of low temperature and gibberellin on *VRN1* and GA biosynthesis genes expression levels

In *Arabidopsis*, flowering is promoted by exogenous application of GA, which regulates the meristem identity genes *AP1*, *CAL* and *LFY* in a positive manner (Andrés and Coupland, 2012). In barley, *VRN1*, the orthologue of *AP1*, has a central role in controlling the transition from vegetative to reproductive stage (Danyluk *et al.*, 2003; Trevaskis *et al.*, 2003; Yan *et al.*, 2003, 2004a). Here, I wanted to test if *VRN1* was affected by temperature and applied GA, and if there was a correlation with the data obtained in the flowering time experiments. Moreover, I wanted to understand if a reduction of GA biosynthesis, as it happens in the *grd5* mutants, could cause a different expression of *VRN1* gene and, eventually, could cause a different response of this gene to applied GA. For these experiments, I grew barley seedlings for one week at 15°C or at 8°C, I treated them with GA_3, and then I collected 3 leaves stage seedlings 0, 2, 4, 6 hours after the treatment.

3.1. *VRN1* expression depends on temperature and gibberellin

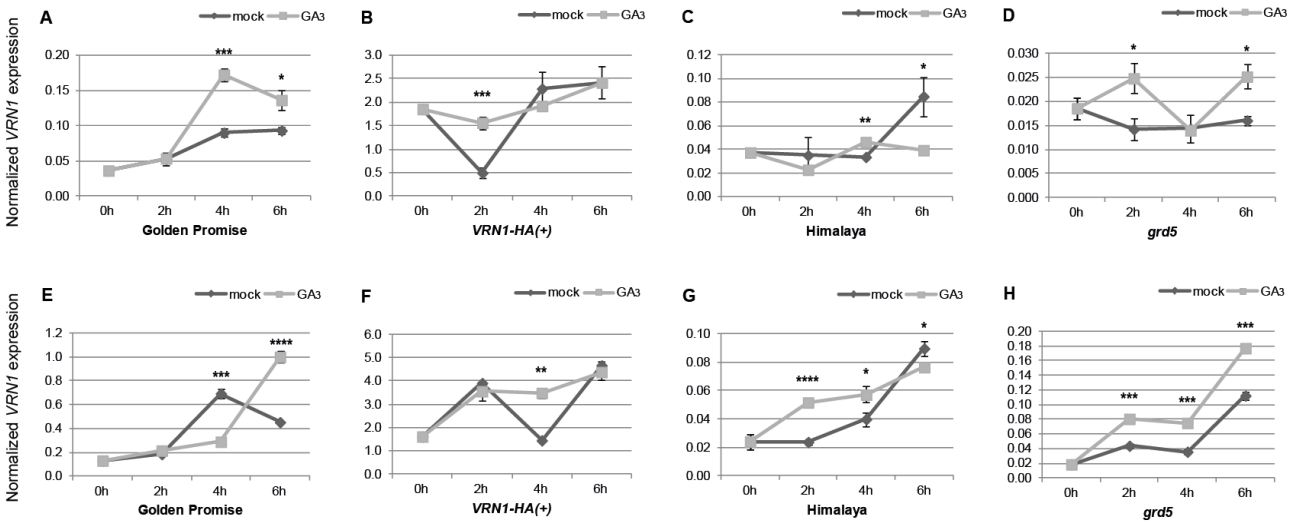

Figure 13. *VRN1* expression levels upon GA_3 treatment. (A-D) 15°C; (E-H) 8°C. Data are normalized to *ACTIN*. Each point shows the mean and the standard error of three biological replicates. Asterisks indicate *P*-values of Student's *t*-test. (*P<0.05; **P<0.01; ***P<0.001; ****P<0.0001).

In Golden Promise seedlings grown at 15°C, a positive regulation of the *VRN1* transcript levels by exogenous applications of GA was observed. In particular, the strong and positive regulation is evident starting from 4 hours after the treatment. At 6 hours, the effect of applied GA decreased, but the upregulation of *VRN1* was still significant (Figure 13A). In *VRN1-HA(+)* transgenic lines, an initial positive regulation of *VRN1* expression was observed, but the effect of applied GA was limited during the time, because there was no difference in *VRN1* transcript levels between mock- and GA_3- treated seedlings at 4 hours after the treatment (Figure 13B). These results correlate in a positive manner with the data obtained in the flowering time experiments. In Golden Promise, in normal growth conditions, applied GA slightly accelerated the heading date, and slightly upregulated *VRN1*. Similarly, the lack of upregulation of *VRN1* expression in *VRN1-HA(+)* by applied GA reflected, on the long term (6 hours), the non-responsiveness of the transgenic lines to GA treatments in terms of reduction of the heading date.

In mock-treated Himalaya seedlings, *VRN1* transcript levels were very similar to those found in Golden Promise grown at 15°C. Their trend over time was also similar, it was possible to observe an upregulation of *VRN1* expression between 0 and 6 hours in both cultivars (Figures 13A and 13C). Despite these similarities, Himalaya plants flowered earlier than Golden Promise. Moreover, in Himalaya seedlings, applied GA decreased *VRN1* expression levels at 6 hours after the treatment (Figure 13C). *VRN1* transcript levels in response to gibberellin treatment in *grd5* mutant seedlings showed an oscillatory trend over time, with a later upregulation 6 hours after the

treatment (Figure 13D). This result did not reflect the non-responsiveness of *grd5* to applied GA in terms of heading date at 15°C. However, *VRN1* levels in the mutant line were lower than those detected in Himalaya (Figures 13C and 13D), and the mutants flowered later than Himalaya plants, suggesting that a functional gibberellin signaling pathway is required for a proper flowering.

In Golden Promise seedlings grown at 8°C, the positive regulation of *VRN1* by exogenous GA applications took place later, at 6 hours after the treatment, and it was stronger in comparison to 15°C (Figure 13E). This result reflects the difference in the heading date between mock- and GA_3-treated Golden Promise plants at 8°C than at 15°C, suggesting that Golden Promise plants were more sensitive to applied GA in cold temperatures. Likewise, *VRN1-HA(+)* lines grown at 8°C showed a later *VRN1* upregulation at 4 hours after the treatment, in comparison to the upregulation visible at 2 hours at 15°C, and even at 8°C, applied GA did not have any further effect later in time (Figure 13F).

In Himalaya plants, applied GA had an initial promoting effect on the *VRN1* transcript levels in low temperature conditions, but 6 hours after the treatment, exogenous applications of gibberellin did not have any further effect (Figure 13G). As already mentioned, VRN1 has a pivotal role in promoting flowering, and the lack of its further upregulation by GA later in time is consistent with the lack of difference in the heading date of Himalaya plants when subjected to mock or GA treatments. Whereas, in *grd5* mutant seedlings, *VRN1* transcript levels were strongly upregulated upon GA treatments (Figure 13H), and this result fully reflects the faster flowering of GA_3-treated *grd5* plants in comparison to the mock-treated ones.

3.2. Temperature-dependent regulation of GA biosynthesis gene expression levels

In higher plants, and in barley, the bioactive forms of gibberellin are GA_1, GA_3, GA_4, and GA_7. Production of bioactive GA and their activation are under the control of GA20ox, GA3ox, and GA2ox enzymes (Hedden and Phillips, 2000; Olszewski *et al.*, 2002; Spielmeyer *et al.*, 2004). The deactivation is important for an effective regulation of the concentration of bioactive GA in plants (Yamaguchi, 2008). Moreover, temperature has an effect on the behavior of GA biosynthesis genes in different species. In *Arabidopsis*, low temperatures upregulate *GA20ox* genes and downregulate *GA2ox* genes (Lee *et al.*, 2005); in wheat, the growth in low temperatures is promoted by exogenous applications of gibberellin (Tonkinson *et al.*, 1997); and in citrus the increase in temperature is linked to an increase in the transcript levels of *GA20ox* genes (Vidal *et al.*, 2003). For these reasons, and for the fact that currently it is unknown if in barley the GA biosynthesis genes are temperature-regulated, I want to understand if also in barley the GA

biosynthesis genes are subjected to temperature regulation. For this purpose I grew barley seedlings at 15°C or at 8°C for one week, and then I checked the variation of the expression levels of some of the genes involved in the gibberellin biosynthesis and deactivation pathway.

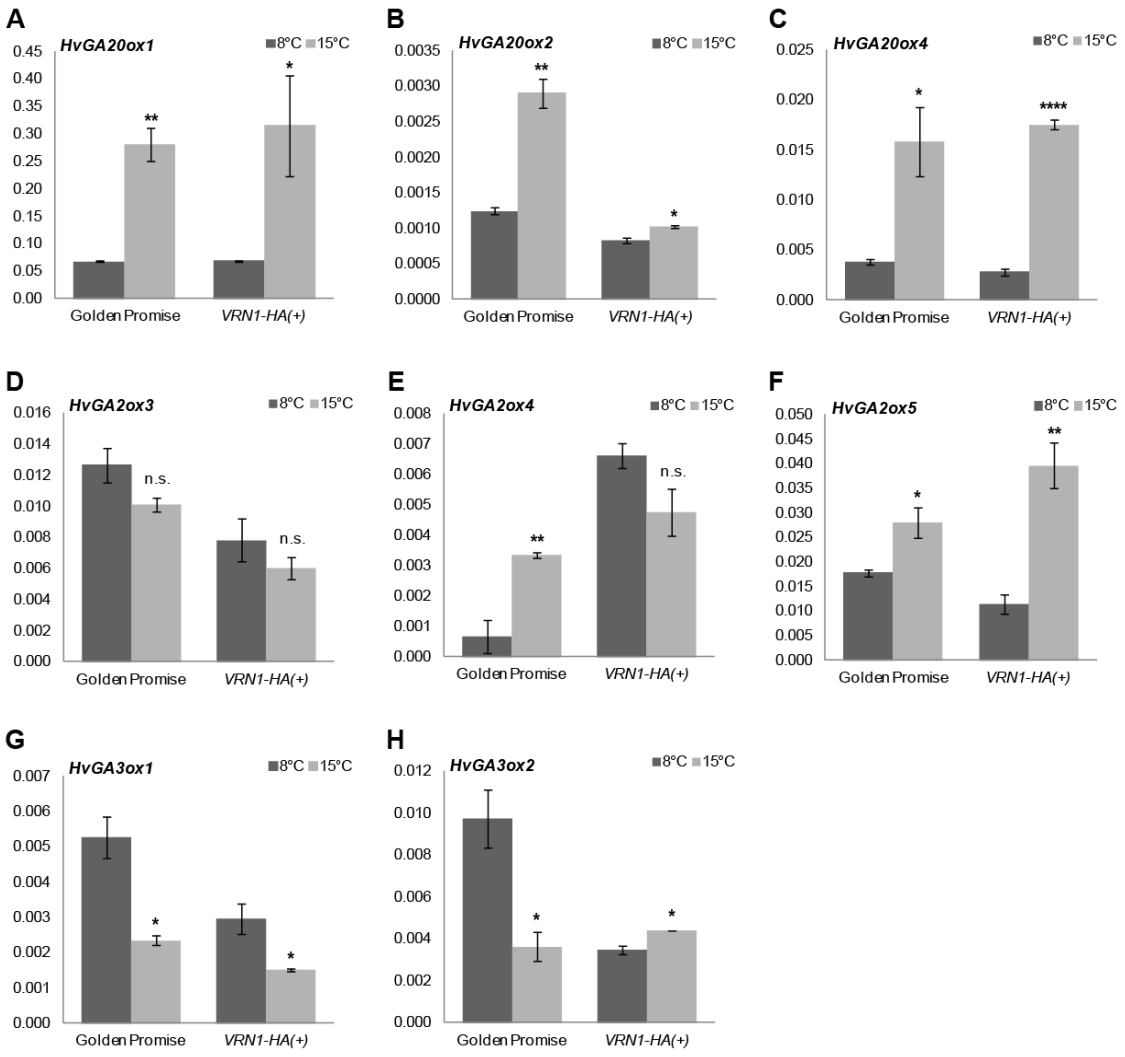

Figure 14. GA biosynthesis gene expression levels in Golden Promise and *VRN1-HA(+)* at 8°C and at 15°C. Data are normalized to *ACTIN*. Asterisks indicate *P*-values of Student's *t*-test. (*P<0.05; **P<0.01; ****P<0.0001; n.s., not significant).

Increasing the temperature from 8°C to 15°C had a similar effect on the expression levels of the GA biosynthesis genes in Golden Promise and *VRN1-HA(+)*. An increase in *HvGA20ox* genes transcript levels was observed (Figure 14A-C), and an increase of *HvGA2ox5* expression was observed (Figure 14F). There were no differences in *HvGA2ox3* expression (Figure 14D), and *HvGA2ox4* increased its expression in Golden Promise but not in *VRN1-HA(+)* (Figures 14E). *HvGA3ox1* decreased its expression in Golden Promise and in *VRN1-HA(+)*, but *HvGA3ox2* was strongly upregulated in Golden Promise and slightly downregulated in *VRN1-HA(+)* (Figure 14G

and 14H). These results suggest the presence of a temperature regulation of the genes involved in the synthesis of gibberellin, in a mechanism needed to control the appropriate levels of active GA during plant growth. This mechanism ensures that the endogenous concentration of bioactive GA is maintained within certain limits, to avoid the inhibition of the GA responses.

It was also possible to observe a difference between Golden Promise and *VRN1-HA(+)* in the GA biosynthesis gene expression levels, in cold as well as in normal temperature growth conditions. A high *VRN1* expression is likely the cause of these differences. This suggests that an elevated amount of VRN1 can, directly or indirectly, affect the expression levels of the GA biosynthesis genes as well as the flowering time and the meristem development, confirming its key role in the regulation of the transition from vegetative stage to reproductive stage.

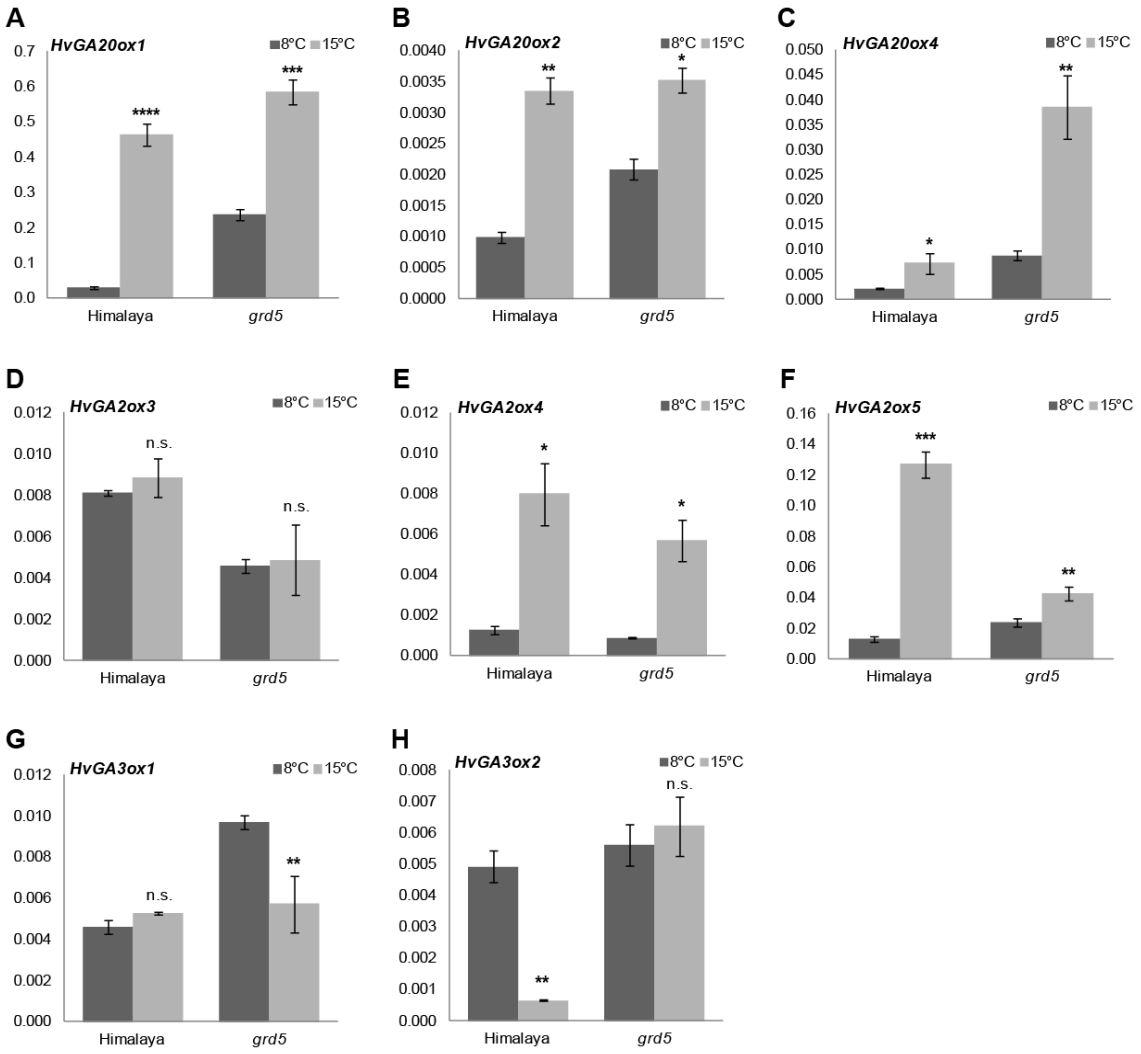

Figure 15. GA biosynthesis gene expression levels in Himalaya and *grd5* at 8°C and at 15°C. Data are normalized to *ACTIN*. Asterisks indicate *P*-values of Student's *t*-test. (*P<0.05; **P<0.01; ***P<0.001; ****P<0.0001; n.s., not significant).

The behavior of the GA biosynthesis genes between Himalaya and *grd5* appeared to be very similar. The increase of temperature from 8°C to 15°C caused an upregulation of *HvGA20ox* genes, *HvGA2ox4*, and *HvGA2ox5*, whereas *HvGA2ox3* did not show any difference in its transcript levels (Figures 15A-F). The *HvGA3ox* genes showed an opposite behavior: in Himalaya *HvGA3ox1* did not change its transcript level, whereas *HvGA3ox2* strongly decreased its expression when the temperature increases; in *grd5*, *HvGA3ox1* strongly decreased its level responding to an increase of temperature, whereas *HvGA3ox2* did not change its expression (Figures 15G and 15H). Nevertheless, the expression levels of these genes is very different in Himalaya and *grd5*, despite of that they showed a similar behavior in response to temperature. Since in *grd5* mutant the GA biosynthesis pathway is impaired, the endogenous amount of bioactive GA is reduced (Helliwell *et al.*, 2001), and since in *Arabidopsis* the GA biosynthesis genes are subjected to a GA-dependent regulation (Olszewski *et al.*, 2002), it is possible to conclude that this regulation also takes place in barley.

A comparison between Himalaya and Golden Promise seedlings highlighted the differences in the expression levels of some of the GA biosynthesis genes. In Himalaya there was an upregulation of *HvGA20ox1*, *HvGA3ox1*, *HvGA2ox4*, and *HvGA2ox5*, and a downregulation of *HvGA20ox4*, *HvGA3ox2*, whereas *HvGA20ox2* and *HvGA2ox3* did not show any differences in their expression levels. These results suggest that in Himalaya, the presence of an active *Ppd-H1* allele affects the expression of the genes involved in the GA synthesis, causing an increase of the endogenous amount of bioactive GA. This correlates with the accelerated flowering time of Himalaya plants in comparison to Golden Promise, and it strongly suggests that both the GA signaling pathway and the photoperiod pathway promote flowering.

3.3. Gibberellin-dependent regulation of GA biosynthesis gene expression levels

In *Arabidopsis*, most of the *GA20ox* genes and *GA3ox* genes, required for the production of bioactive gibberellins, are downregulated by GA treatment, whereas the *GA2ox* genes, required for the conversion of active GAs to inactive catabolites, are upregulated by applied GA. This feedback regulation is important to control the endogenous amount of bioactive GA plants (Olszewski *et al.*, 2002). I wanted to understand if this feedback regulation also takes place in barley and tested the effect of exogenous application of GA in low temperature on the expression levels of the genes involved in gibberellin synthesis and catabolism. For this purpose, I grew barley seedlings at 15°C and at 8°C, for one week, then I treated them with GA_3, and I collected samples at 0, 2, 4, and 6 hours after the treatment.

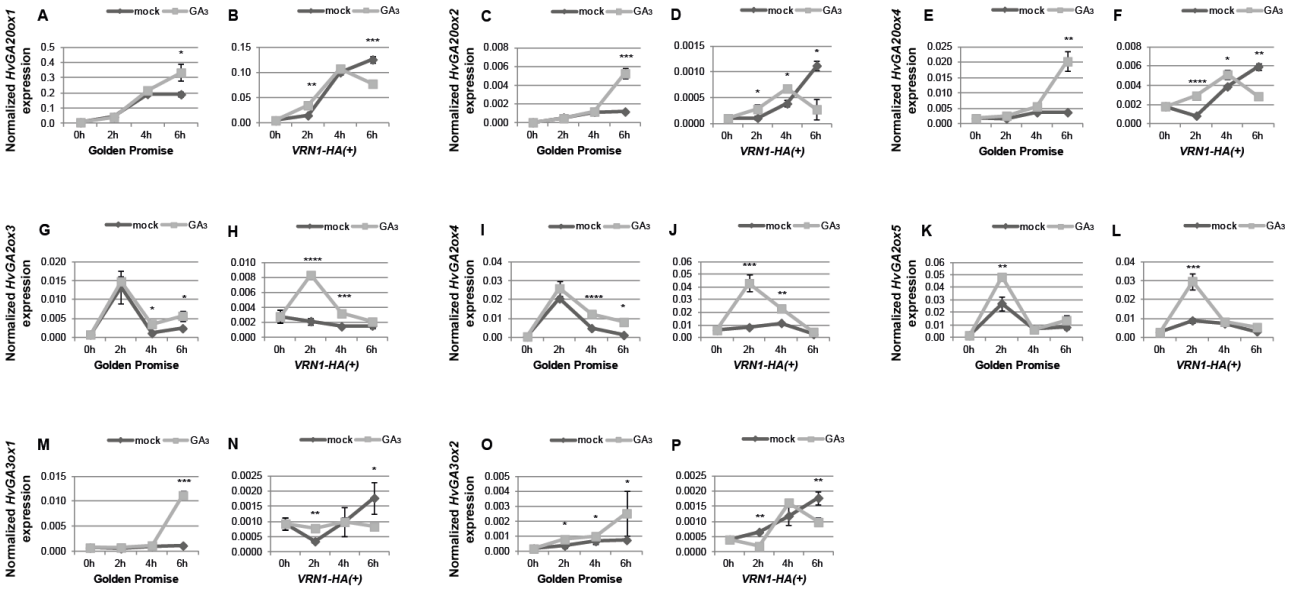

Figure 16. GA biosynthesis gene expression levels in Golden Promise and *VRN1-HA(+)* at 15°C upon GA_3 treatment. Data are normalized to *ACTIN*. Each point shows the mean and the standard error of three biological replicates. Asterisks indicate *P*-values of Student's *t*-test. (*P<0.05; **P<0.01; ***P<0.001; ****P<0.0001).

In normal growth temperatures (15°C) in Golden Promise seedlings, the *HvGA20ox* genes showed an initial non-response to exogenous GA application, and a strong and late increase in their transcript levels, between 4 and 6 hours after the treatment. In *VRN1-HA(+)* transgenic plants, these genes showed a varied response to applied GA. *HvGA20ox1* had a similar behavior to the one found in Golden Promise, *HvGA20ox2* showed a slight but constant initial upregulation and later on is strongly downregulated by GA_3, whereas *HvGA20ox4* showed a fast upregulation, followed by a downregulation upon GA treatment (Figures 16A-F). However, in Golden Promise, *HvGA2ox5* was strongly upregulated during the initial phases of GA_3 treatment, and already 2 hours after the treatment it was possible to see a substantial increase in its transcript level; while *HvGA2ox3* and *HvGA2ox4* showed a later upregulation, after 4 hours. A similar effect was seen in *VRN1-HA(+)* plants, a very rapid initial upregulation of the *HvGA2ox* genes, followed by a downregulation to the mock level (Figures 16G-L). Additionally, in Golden Promise there was an upregulation of *HvGA3ox1* and *HvGA3ox2* respectively between 4 and 6 hours and between 2 and 4 hours after GA_3 treatment. In *VRN1-HA(+)*, the *HvGA3ox* genes responded faster to temperature than to GA application, which downregulated later their expression (Figures 16M-P). This suggests that, over time, when the *HvGA20ox* genes did not appear to be affected by exogenous gibberellin applications and *HvGA2ox* genes were strongly upregulated, *HvGA3ox2* was rapidly upregulated to compensate the deactivation activity of *HvGA2ox* genes, while *HvGA3ox1* was delayed up to work in parallel with the *HvGA20ox* genes, that were upregulated later in time, to prevent unnecessary accumulation of precursors and to have an adequate bioactive GA level.

Taken together these results seem to suggest the existence of a feedback mechanism with the aim to keep gibberellin within levels needed to maintain the developmental responses to this hormone active.

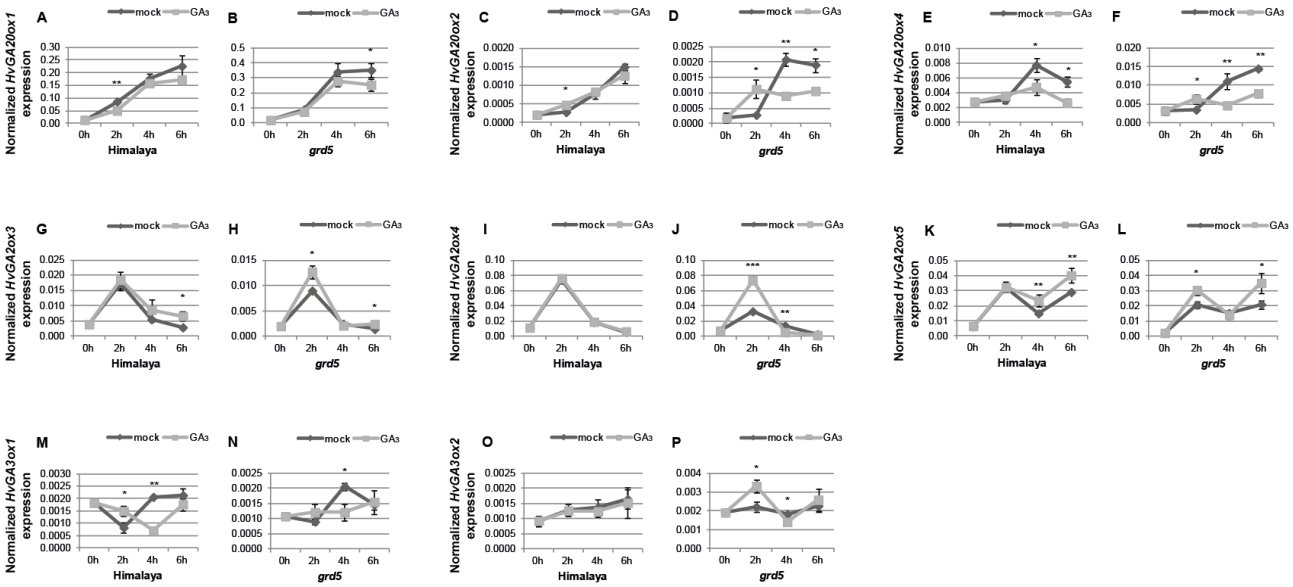

Figure 17. GA biosynthesis gene expression levels in Himalaya and *grd5* at 15°C upon GA_3 treatment. Data are normalized to *ACTIN*. Each point shows the mean and the standard error of three biological replicates. Asterisks indicate *P*-values of Student's *t*-test. (*P<0.05; **P<0.01; ***P<0.001).

In Himalaya plants, exogenous applications of gibberellin in normal growth conditions (15°C) had a weak and early effect on *HvGA20ox1* and *HvGA20ox2*, at 2 hours after the treatment it was possible to observe, respectively, a downregulation and an upregulation of their expression levels, but later in time the transcript levels of both genes were not different to that found in mock-treated seedlings. *HvGA20ox4* instead, was downregulated at 4 and 6 hours after the treatment (Figures 17A, 17C, and 17E). In Himalaya seedlings it was also possible to observe an increase of the transcript levels of *HvGA2ox3* and *HvGA2ox5*, respectively at 6 and 4 hours after the treatments; whereas *HvGA2ox4* did not show any alterations in its expression (Figures 17G, 17I, and 17K). *HvGA3ox1* showed an oscillatory trend of its expression, whereas *HvGA3ox2* was not influenced by exogenous application of gibberellin (Figures 17M and 17O). Therefore, it was possible to conclude that, on the long term, there were differences on the expression levels in only three out of eight genes involved in the GA biosynthesis pathway checked in Himalaya.

The differences in the transcript levels were slight and they suggested that the amount of endogenous GA was constant and similar to mock-treated seedling, and that the GA developmental responses were not enhanced. This result is in line with the fact that Himalaya plants treated with gibberellin did not accelerate their flowering time.

In *grd5* mutant seedlings the behavior of the GA biosynthesis genes at 15°C upon gibberellin treatment was similar to that observed in Himalaya. The *HvGA20ox* genes showed a downregulation later in time, in between 4 and 6 hours after the treatment (Figures 18B, 18D, and 18F). *HvGA2ox3* and *HvGA2ox5* showed a slight but significant upregulation at 2 and 6 hours after the treatment, whereas *HvGA2ox4* rapidly increased its transcript level, but later in time its level decreased to that detected in mock-treated seedlings (Figures 18H, 18J, and 18L). The *HvGA3ox* genes showed an early response to applied GA, increasing and decreasing, respectively, their levels, but at 6 hours after the treatment their expression decreased to that detected in mock-treated seedlings (Figures 18N, and 18P). These results suggest that *grd5* plants, in presence of exogenous gibberellin, reduce the endogenous production of bioactive GA.

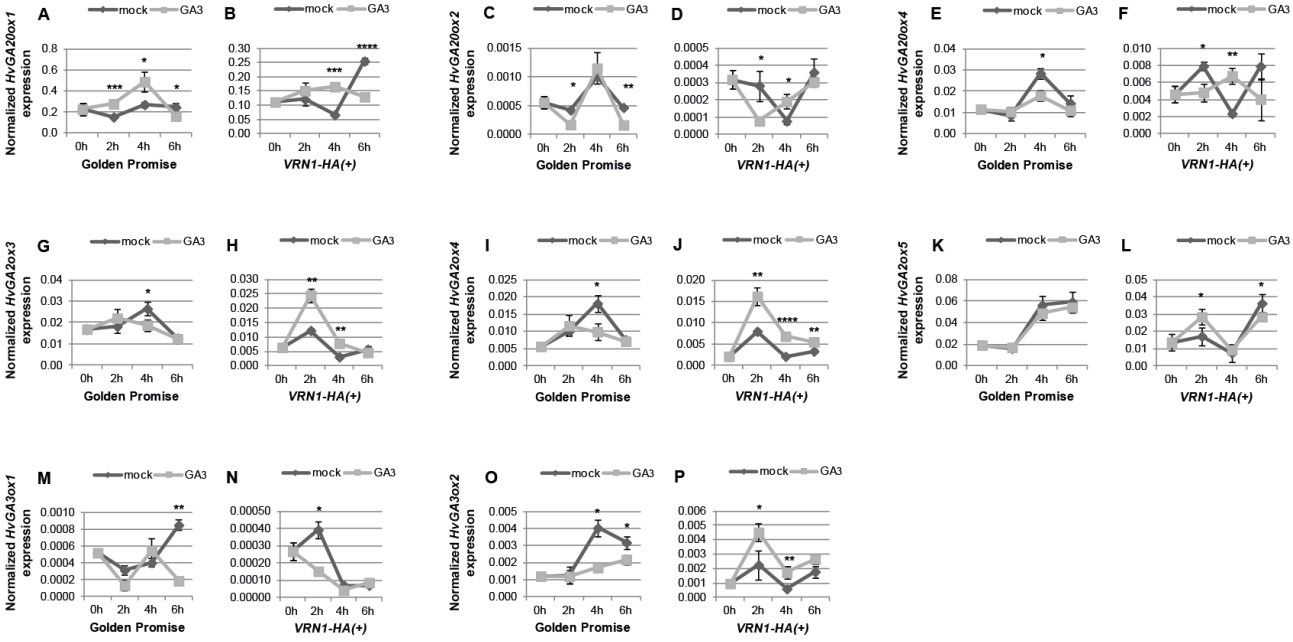

Figure 18. GA biosynthesis gene expression levels in Golden Promise and *VRN1-HA(+)* at 8°C upon GA_3 treatment. Data are normalized to *ACTIN*. Each point shows the mean and the standard error of three biological replicates. Asterisks indicate *P*-values of Student's *t*-test. (*P<0.05; **P<0.01; ***P<0.001; ****P<0.0001).

In low temperature growth conditions, in mock-treated Golden Promise seedlings, *HvGA20ox1* did not change its transcript level (Figure 18A); *HvGA20ox2*, *HvGA20ox4*, *HvGA2ox3*, and *HvGA2ox4* were subjected to an upregulation 4 hours after the treatment, followed by a decrease in their expression to the level found at 0 hours (Figures 18C. 18E, 18G, and 18I); whereas *HvGA2ox5*, *HvGA3ox1*, and *HvGA3ox2* were upregulated by cold (Figure 18K, 18M, and 18O). Exogenous applications of gibberellin, in Golden Promise seedlings, had the general effect to downregulate the transcript levels of almost all the GA biosynthesis genes checked. In *VRN1-HA(+)* transgenic

plants, the expression pattern of the GA biosynthesis genes in response to low temperature and GA treatments showed differences from Golden Promise. The *HvGA20ox1* gene gradually increased its expression level in response to applied GA, but temperatures seem to have a stronger effect on the long term, upregulating drastically its expression at 6 hours in mock-treated plants (Figure 18B). *HvGA20ox2* and *HvGA20ox4* were strongly and rapidly downregulated by GA_3, whereas in mock-treated plants *HvGA20ox2* showed a later and slight downregulation, and *HvGA20ox4* seemed to respond positively to low temperatures (Figures 18D and 18F). *HvGA2ox3* and *HvGA2ox4* showed a similar expression pattern in response to low temperature and to GA, where applied GA strongly upregulates their expression, whereas *HvGA2ox5* showed an early and slight upregulation of its expression by applied GA (Figures 18G, 18I, and 18K). The *HvGA3ox1* gene was constantly downregulated by exogenous GA, whereas *HvGA3ox2* was upregulated (Figure 18N and 18P).

These results suggest a decline in the production of the precursors of bioactive GA, and an increased production of bioactive GA, probably to maintain GA-regulated developmental responses active in plants in cold temperatures.

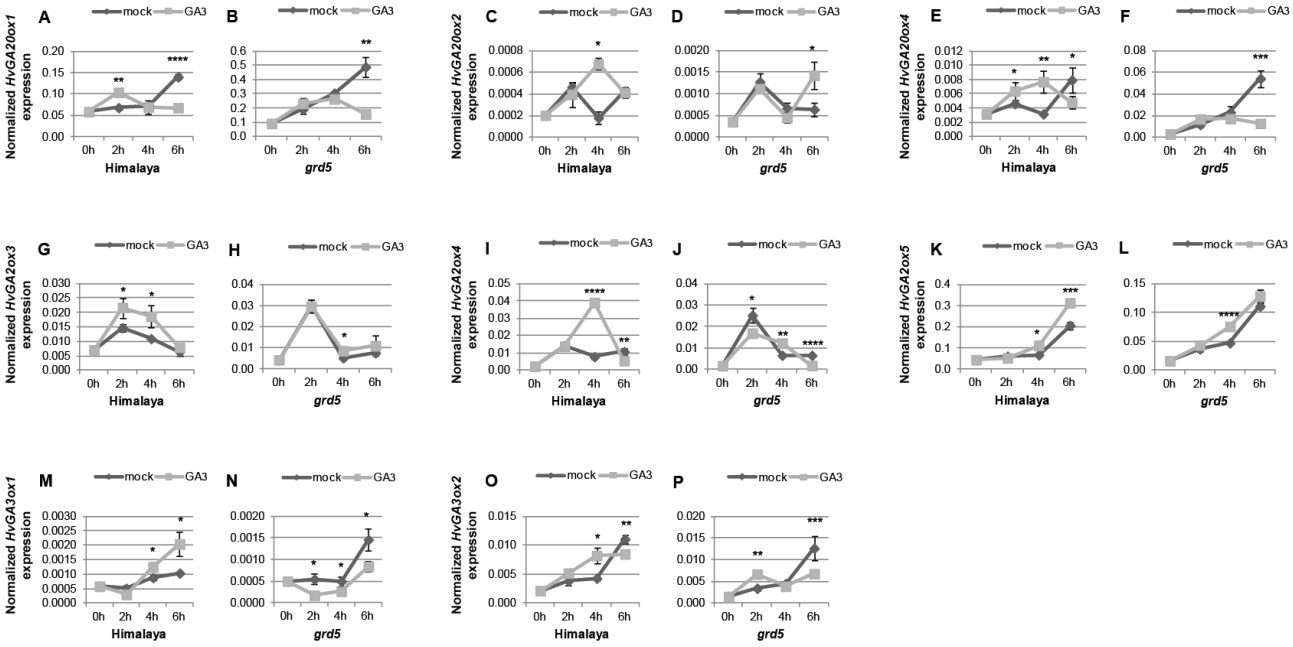

Figure 19. GA biosynthesis gene expression levels in Himalaya and *grd5* at 8°C upon GA_3 treatment. Data are normalized to *ACTIN*. Each point shows the mean and the standard error of three biological replicates. Asterisks indicate *P*-values of Student's *t*-test. (*P<0.05; **P<0.01; ***P<0.001; ****P<0.0001).

In Himalaya seedlings, low temperature strongly upregulated *HvGA20ox1*, *Hv20ox4*, *HvGA2ox5*, and *HvGA3ox2*; the upregulation of *HvGA2ox4* and *HvGA3ox1* was not very strong; whereas

HvGA2ox3 showed an initial upregulation, followed by a downregulation. Differently from Golden Promise, in Himalaya cold temperatures seemed to promote both the production of the precursor of bioactive GA and the production of bioactive GA in a feedback loop mechanism. In response to applied GA, the transcript level of the GA biosynthesis genes changed very rapidly. All the genes checked showed a very fast upregulation in between 2 and 4 hours after the treatment, but on the long term, 6 hours, only *HvGA2ox5* and *HvGA3ox1* keep their expression levels higher than the mock (Figure 19). In *grd5* mutant seedlings, cold temperature strongly upregulated *HvGA20ox1*, *HvGA20ox4*, *HvGA2ox5*, *HvGA3ox1*, and *HvGA3ox2*; whereas *HvGA20ox2*, *HvGA2ox3*, and *HvGA2ox4* slightly increased their transcript levels. Applied GA, in *grd5* mutants, caused a downregulation of *HvGA20ox1*, *HvGA20ox4*, *HvGA2ox4*, *HvGA3ox1*, and *HvGA3ox2*; whereas *HvGA20ox2* increased its levels, and *HvGA2ox3* and *HvGA2ox5* did not change their expression (Figure 19).

These results suggest the presence of a feedback loop mechanism in Himalaya as well as in *grd5* plants in response to exogenous GA, needed to keep the endogenous amount of GA within a range to maintain active the GA responses.

3.4. Time course analysis of GA biosynthesis gene expression levels

To evaluate the short term effect of changes in temperature on GA biosynthesis gene expression in barley seedlings, two shift experiments followed by qRT-PCR were performed. In one experiment, barley seedlings were grown at 15°C for one week, afterwards the temperature was decreased to 8°C; in the second one barley seedlings were grown at 8°C for one week, afterwards the temperature was increased to 15°C. In both experiments, samples were collected 0, 2, 4 and 6 hours after the temperature shift.

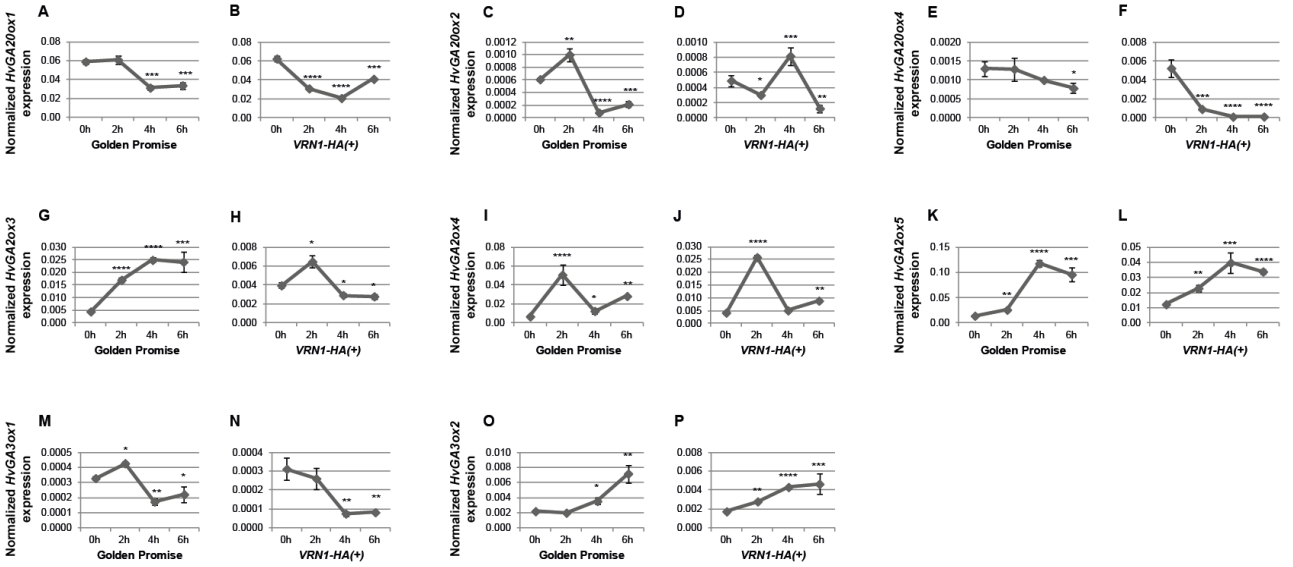

Figure 20. GA biosynthesis gene expression levels in Golden Promise and *VRN1-HA(+)* in a shift experiment where the temperature was decreased from 15°C to 8°C. Data are normalized to *ACTIN*. Each point shows the mean and the standard error of three biological replicates. Asterisks indicate *P*-values of Student's *t*-test. (*P<0.05; **P<0.01; ***P<0.001; ****P<0.0001).

In the shift experiment where barley seedling were grown for one week at 15°C and then the temperature was decreased to 8°C, it was possible to observe a fast response in the transcript levels of the genes involved in the biosynthesis of GA. In Golden Promise and *VRN1-HA(+)* transgenic lines, the genes checked showed a similar behavior in response to changes in temperature. The *HvGA20ox* genes decreased their expression levels few hours after the alteration of the temperature (Figures 20A-F). The two *HvGA3ox* genes tested showed an opposite response: *HvGA3ox1* decreased its expression, whereas *HvGA3ox2* increased its level (Figures 20M-P). This can be explained with the fact that at least one of the enzymes required for the production of bioactive GA, encoded by the *HvGA3ox* genes, has to be active to lead the plants to control the amount of bioactive GA. Moreover, as expected, the *HvGA2ox* genes, in cold temperature, increased their transcript levels; this is in line with our model, which proposes that cold temperature promotes the catabolism of GA (Figures 20I-L). The only exception is *HvGA2ox3*, which increases its expression in Golden Promise and decreases its level in *VRN1-HA(+)* (Figures 20G and 20H).

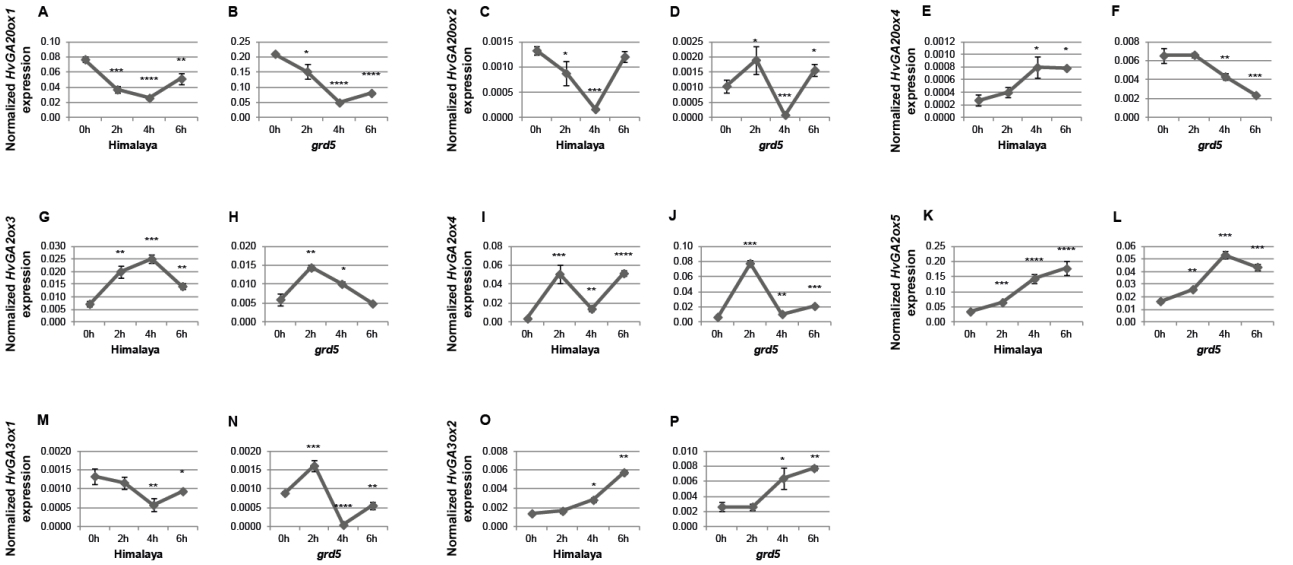

Figure 21. GA biosynthesis gene expression levels in Himalaya and *grd5* in a shift experiment where the temperature was decreased from 15°C to 8°C. Data are normalized to *ACTIN*. Each point shows the mean and the standard error of three biological replicates. Asterisks indicate *P*-values of Student's *t*-test. (*P<0.05; **P<0.01; ***P<0.001; ****P<0.0001).

In a similar way, the behavior of the genes involved in the biosynthesis and catabolism of GA did not show a lot of differences between Himalaya and *grd5*. *HvGA20ox1* decreased its expression level in both lines. *HvGA20ox2* in Himalaya showed a strong downregulation followed by an upregulation at 6 hours after the change in the temperature, reaching its initial expression level; whereas in *grd5* *HvGA20ox2* showed a long term upregulation. *HvGA20ox4* had an opposite behavior, it was upregulated in Himalaya and downregulated in *grd5* (Figures 21A-F). *HvGA2ox3* was upregulated, but on the long term its level decreased. *HvGA2ox4*, *HvGA2ox5* (Figures 21G-L), and *HvGA3ox2* were strongly upregulated after the decrease of the temperature (Figures 21O and 21P). *HvGA3ox1* was downregulated, but in *grd5* it was subjected to an early and very strong upregulation 2 hours after the temperature alteration (Figures 21M and 21N).

These results suggest the presence, in all the genotypes analyzed, of an endogenous regulation of the amount of GA present in plants when the environmental temperatures are not suitable for proper growth and development, and that the regulation implemented by the plant is extremely fast.

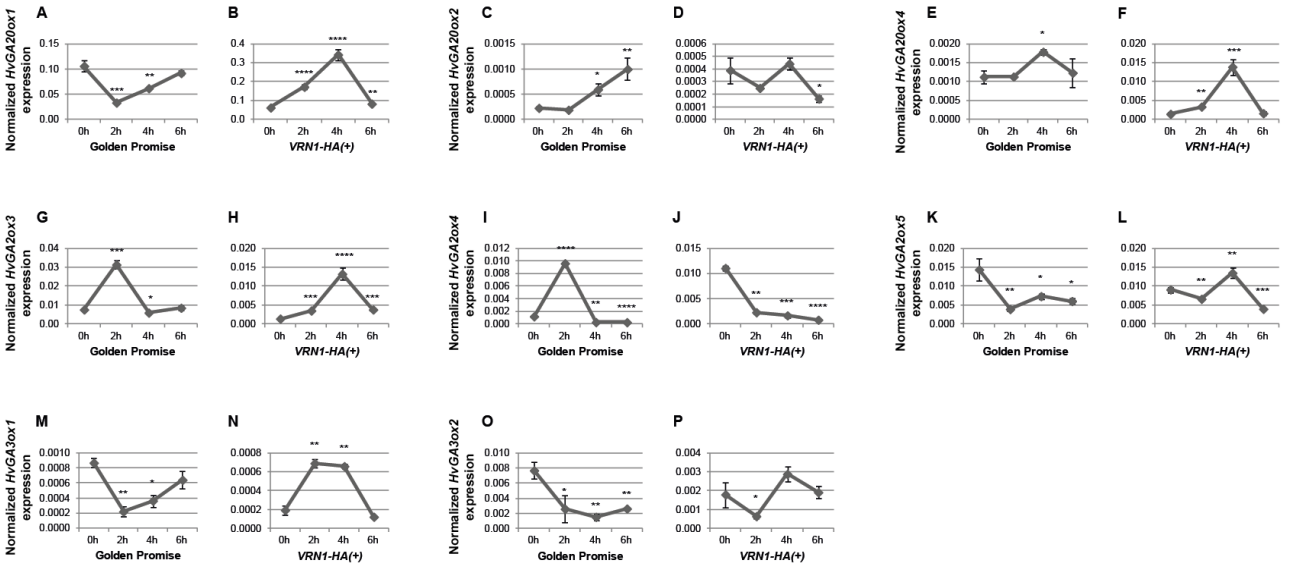

Figure 22. GA biosynthesis gene expression levels in Golden Promise and *VRN1-HA(+)* in a shift experiment where the temperature was increased from 8°C to 15°C. Data are normalized to *ACTIN*. Each point shows the mean and the standard error of three biological replicates. Asterisks indicate *P*-values of Student's *t*-test. (*P<0.05; **P<0.01; ***P<0.001; ****P<0.0001).

Likewise, when barley seedlings grown at 8°C were subjected to a temperature increase, the response of the genes involved in the synthesis of gibberellin was fast. In Golden Promise, *HvGA20ox1* decreased its expression very fast, but 6 hours after the temperature increase, its level reached the initial one; whereas in *VRN1-HA(+)* it was upregulated (Figures 22A and 22B). *HvGA20ox2* was upregulated in Golden Promise and downregulated in the transgenic line (Figures 22C and 22D). There was no change in the expression level of *HvGA20ox4* in Golden Promise; whereas in *VRN1-HA(+)* its expression was subjected to a strong and fast upregulation, followed by a strong downregulation until its initial level (Figures 22E and 22F). *HvGA2ox3* was upregulated in both lines, but in Golden Promise, at 6 hours after the temperature alteration, it was downregulated to its initial level (Figures 22G and 22H). *HvGA2ox4* and *HvGA2ox5* were both downregulated (Figures 22I and 22L). *HvGA3ox1* and *HvGA3ox2* were downregulated in Golden Promise; whereas in the transgenic line, after an early and strong change in their expression, their levels were stabilized to the initial ones (Figures 22M-P).

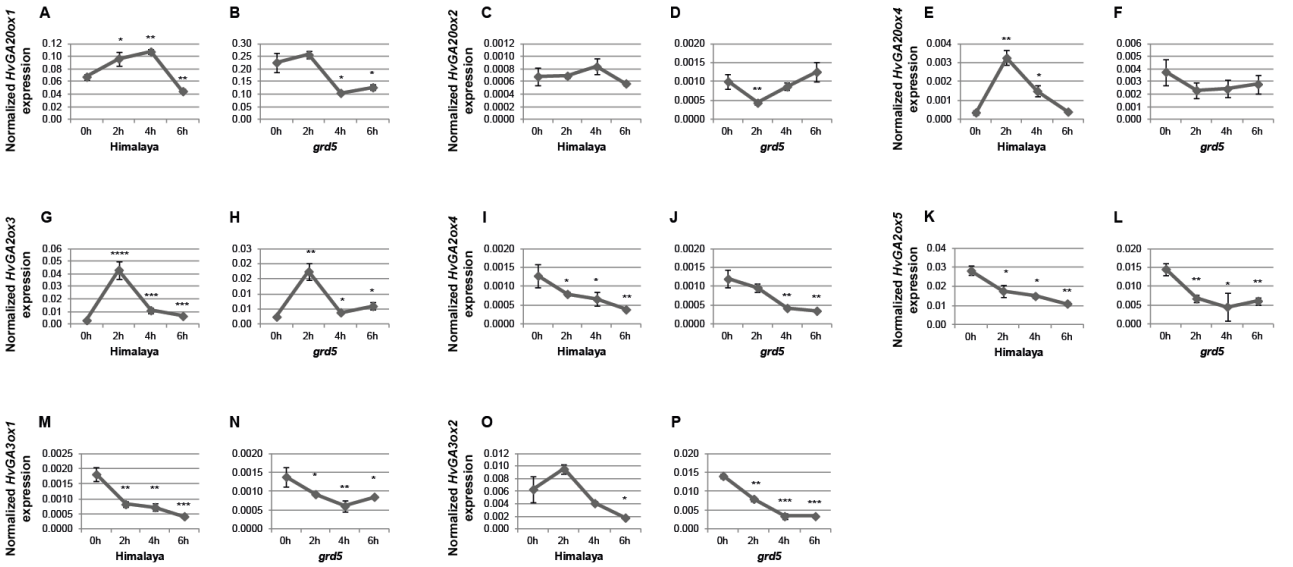

Figure 23. GA biosynthesis gene expression levels in Himalaya and *grd5* in a shift experiment where the temperature was increased from 8°C to 15°C. Data are normalized to *ACTIN*. Each point shows the mean and the standard error of three biological replicates. Asterisks indicate *P*-values of Student's *t*-test. (*P<0.05; **P<0.01; ***P<0.001; ****P<0.0001).

The increase of temperature had a similar effect on the expression of the GA biosynthesis genes in Himalaya and *grd5* mutant. *HvGA20ox1* was downregulated, *HvGA20ox2* and *HvGA20ox4* did not change their transcript levels, but *HvGA20ox4* showed an early and strong upregulation in Himalaya (Figures 23A-F). *HvGA2ox3* was upregulated, whereas *HvGA2ox4* and *HvGA2ox5* were both downregulated (Figures 23G-L). *HvGA3ox1* and *HvGA3ox2* were both downregulated (Figures 23M-P).

In conclusion, these results confirm the presence of a temperature-dependent regulation of the genes involved in the synthesis of GA and that this regulation is very fast, to ensure a proper plant growth and development. Moreover, this correlates with the model proposed here, which hypothesizes a temperature-dependent regulation of flowering through the gibberellin signaling pathway.

4. Interaction analysis between VERNALIZATION1 (VRN1) and SLENDER1 (SLN1)

In *Arabidopsis*, the expression of *AP1* is downstream the flowering promoting pathways and AP1 overexpression causes early flowering (Mandel and Yanofsky, 1995). Moreover, in our laboratory it has been shown that AP1 is repressed by direct interactions with DELLAs. In barley, VERNALIZATION1 (VRN1), the closest homologue of AP1, is the master regulator of flowering time (Distelfeld *et al.*, 2009), and its possible interaction with SLN1, the barley DELLA protein, is not known. Then, a yeast two hybrid assay and a GST and HA pull-down assays were performed to test if an interaction between VRN1 and SLN1 from barley takes place.

4.1. VRN1 and SLN1 do not interact *in vivo* in yeast

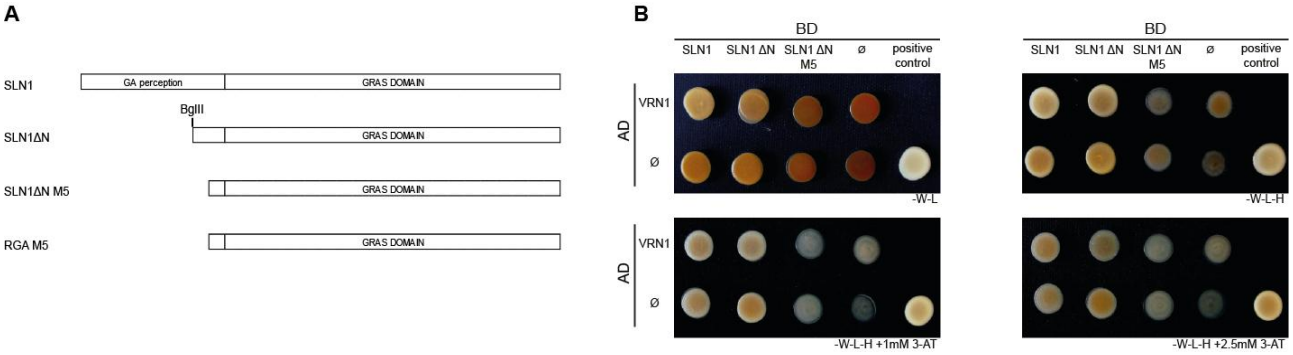

Figure 24. VRN1 and SLN1 do not interact *in vivo* in yeast. (A) Schematic presentation of different truncation constructs of SLN1 in comparison to RGA and GAI M5 from *Arabidopsis*. (B) Yeast two hybrid analysis of SLN1-AD with VRN1-BD. Transformants were plated on synthetic drop-out (SD) medium lacking leucine and tryptophane (-W-L) and on SD medium lacking leucine, tryptophane and histidine (-W-L-H) supplemented with different concentrations of 3-AT to test for their auxotrophic growth.

The first assay I performed to test if VRN1 and SLN1 were able to interact *in vivo*, was a yeast two hybrid assay. First, I cloned the full length ORF of VRN1 into pGATD7 and pGBKT7 vectors. The SLN1 ORF was synthesized by Eurofins MWG Operon, due to the difficulties to clone it, with the codon optimization for the expression of this protein in yeast.

The codon usage bias is defined as differences in the frequency of occurrence of synonymous codons in coding DNA. The excess in the number of codons allows many amino acids to be encoded by more than one codon, and the genetic code of different organisms are often inclined towards using one of the several codons that encode the same amino acid over the others. Optimal codons help to reach faster translation rates and high accuracy.

Since it is known that DELLA proteins from *Arabidopsis* are able to autoactivate in yeast, I also created two truncated versions of SLN1 without the N-terminal domain (Figure 24A). For this

purpose, I created a silent site for the restriction enzyme BglII upstream of the DELLA motif. In this way, once I cloned the full length *SLN1* into the yeast vector, with the appropriate restriction enzymes, I could obtain the *SLN1ΔN* truncated version. Unfortunately, even without the DELLA motif, SLN1 was still able to autoactivate in yeast (Figure 24B). Then, I aligned the full length coding sequence of *SLN1* and its truncated version with the *RGA* M5 version from *Arabidopsis*. The M5 version of *RGA* is a truncated version, which lack of the DELLA and the TVHYNP motifs and shows reduced autoactivation in yeast (Hou *et al.*, 2010; Gallego-Bartolomé *et al.*, 2012). I could find that *SLN1ΔN* contained 66 nucleotides more in the DELLA motif in comparison to *RGA* M5. Thus, I generated a second truncated version of *SLN1*, named *SLN1ΔN* M5, where the N-terminal domain was completely deleted (Figure 24A). In this case, the new truncated version of SLN1 lost the autoactivation activity but also lost the putative interaction with VRN1 (Figure 24B). In conclusion, it was not possible to see an *in vivo* interaction between SLN1 and VRN1 with the yeast two hybrid assay.

4.2. VRN1 and SLN1 do not interact *in vitro*

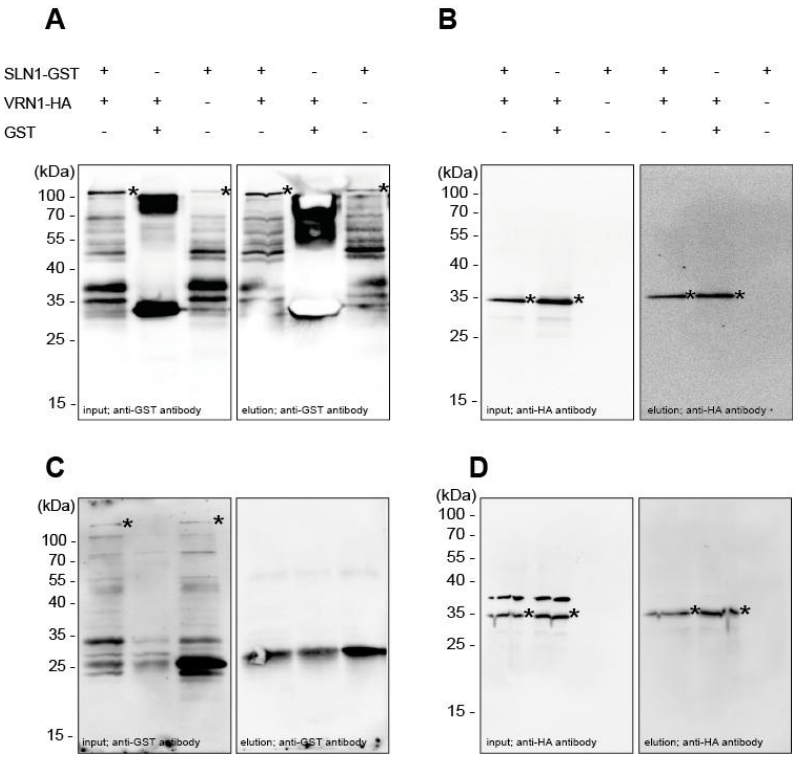

Figure 25. VRN1 and SLN1 do not interact *in vitro.* (A-B) GST pull-down (A) immunoblot analysis with anti-GST antibody; (B) immunoblot analysis with anti-HA antibody. (C-D) HA pull-down. (C) immunoblot analysis with anti-GST antibody; (D) immunoblot analysis with anti-HA antibody. Asterisks indicate in (A-C) SLN1-GST; (B-D) VRN1-HA. After incubation, GST and HA pulled-down beads were analyzed on immunoblot using an anti-HA, anti GST, and anti-SLN1 antibodies.

The yeast two hybrid assay was not positively conclusive, and then an *in vitro* binding assay was performed. For this assay, SLN1-GST fusion protein and GST alone were expressed in and purified from *E. coli*, whereas for the production of VRN1 protein, the TnT® Coupled Wheat Germ Extract System was used. To estimate the purity and to determine the concentration of the purified proteins, proteins were subjected to SDS-PAGE and detected by CBB staining. GST-fused SLN1 or GST were bound to GST beads and then incubated with VRN1. GST was used as negative control. The GST pulled-down samples were subjected to immunoblot analysis with anti-GST and anti-HA antibodies, to detect SLN1-GST fusion protein and VRN1-HA fused protein, respectively. The immunoblot analysis with anti-GST antibody (Figure 25A) revealed that SLN1-GST was correctly pulled-down, meaning that it was bound to the GST beads as shown by the bands highlighted with asterisks in the elution in Figure 26A. However, the immunoblot analysis with anti-HA antibody (Figure 25B) revealed that VRN1-HA was able to bind SLN1-GST, but also GST beads, as revealed by the second band highlighted by an asterisk in the elution in Figure 25B. Then, the putative VRN1-SLN1 interaction, shown by the first band highlighted by an asterisk in the elution in Figure 25B, is not necessarily an interaction. To overcome this problem, an HA immunoprecipitation was performed with HA beads. The HA pulled-down samples were subjected to immunoblot analysis with anti-GST, and anti-HA antibodies, to detect SLN1-GST fused protein and VRN1-HA fused protein, respectively. The immunoblot analysis with anti-HA antibody (Figure 25D) revealed that VRN1-HA was correctly pulled-down, meaning that it was bound to the HA beads as shown by the bands highlighted with asterisks in the elution in Figure 25D. However, the immunoblot analysis with anti-GST antibody (Figure 25C) revealed that SLN1-GST does not bind VRN1-HA, because of the lack of bands corresponding to SLN1-GST in the elution in Figure 25C. In conclusion, I could not show an *in vitro* interaction between SLN1 and VRN1 with the pull down assay.

DISCUSSION

Plants have to synchronize the timing of their flowering when environmental conditions are appropriate. Once plants undergo this transition, they are committed to flower and have evolved several different but interconnected mechanisms to synchronize the flowering time with environmental cues. In barley, temperature and day length have a strong influence on flowering and the capability of the plant to perceive and respond to these signals is controlled by the vernalization pathway and the photoperiod pathway, and their interactions have been well characterized, but the role of gibberellin in this process is poorly understood.

1. Flowering time is affected by low temperature and gibberellin

In all the genotypes analyzed, it was clear that cold conditions have a significant and strong effect delaying the time to head. In low temperature conditions, plants need to slow down all the developmental processes and, in particular, they need to delay the reproductive development in order to ensure a normal and complete development of the reproductive organs, the spikelet, which are fundamental for the propagation of the species, and prevent damage in low temperatures.

In Golden Promise this could be explained by the fact that, at 15°C, the endogenous GA level is sufficient to promote the degradation of the majority of SLN1, leading the plants to flower properly and in the correct time; therefore applied GA does not have a lot of substrate to work on, and the plants do not further reduce their flowering time in response to exogenous GA. In other words, plants do not have the need of extra GA to ensure the proper flowering and flower development when environmental conditions are adequate for growth. Another explanation could be that exogenous GA treatment does not have an effect on the *VRN1* transcript level. Then, without having any significant variation in *VRN1* levels, with or without GA, the flowering time is not significantly affected. As already mentioned above, the presence of GA stimulates the DELLAs degradation and, according to my model, the putative interaction between SLN1 and VRN1 that should block or inhibit the flowering, should not take place, leaving VRN1 to promote the flowering. Then, in *VRN1-HA(+)* lines, it is expected an early flowering phenotype upon GA treatments, but that is not the case. The elevated amount of *VRN1* is already enough to promote the flowering, even causing an earlier flowering phenotype, overcoming the repressive effect of SLN1.

Himalaya and *grd5* plants show no response to applied GA in normal temperature growth conditions. Furthermore, Himalaya plants flower earlier than Golden Promise, whereas *grd5* mutants show a heading date very similar to Golden Promise. The presence of an active *Ppd-H1*

allele leads Himalaya and *grd5* plants to be sensitive to photoperiod, explaining the fact that Himalaya plants flower earlier than Golden Promise. The *grd5* mutants have an impaired GA signaling pathway, and this can explain their delayed heading date in comparison to Himalaya and, at the same time it also explain their similarity to Golden Promise in the flowering time. These results suggest that the presence of a functional photoperiod pathway is enough to promote and accelerate flowering only together with a functioning GA signaling pathway.

Plants grown in low temperatures are more sensitive to applied GA. According to my model, in cold, when endogenous GA are degraded and DELLA proteins accumulate, delaying growth and flowering, exogenous application of GA stimulate the DELLAs degradation process to a level that is sufficient to promote flowering.
Golden Promise plants show a significant reduction in the heading date when treated with gibberellin, and this reduction is more consistent at 8°C than at 15°C. In cold temperatures, *VRN1-HA(+)* transgenic lines still flower earlier than Golden Promise, because of the fact that high basal expression level of VRN1 can overcome the delay in growth and flowering due to an accumulation of DELLA proteins. The non-responsiveness of these plants to exogenous GA application can be due to a saturation in the GA responses. These results suggest that, in the case of *VRN1-HA(+)* transgenic lines a genotype effect in response to cold and not a GA effect was observed, and this is probably linked to the fact that high endogenous expression levels of *VRN1* leads the transgenic plants to flower faster than the wild type plants, independently of applications of exogenous GA.

Himalaya plants show an early flowering phenotype in comparison to Golden Promise and exogenous application of GA do not further accelerate their heading date at 8°C. It is possible to conclude that, even in low temperature growth conditions, the effect of photoperiod and a functional GA signaling pathway on flowering time is so strong that GA treatments do not have any further influence on the acceleration of this process. However, the *grd5* mutant, in the Himalaya background, shows a response to applied GA only in low temperature. This mutant is known to accumulate *ent*-kaurenoic acid in grains, but the GA_{12} levels are not very affected, having a similar content than Himalaya plants (Helliwell *et al.*, 2001). The accumulation of *ent*-kaurenoic acid might affect the flowering of these plants, because mock-treated *grd5* plants flower later than Himalaya, and the time to head is comparable to that measured for Golden Promise. Moreover, *grd5* mutants are very sensitive to applied GA, their heading date is significantly reduced when GA is applied. This can be explained by the fact that the mutant lines have an impaired GA signaling pathway but, differently from Golden Promise, they are sensitive to photoperiod, and the combination between photoperiod sensitivity and GA application significantly reduces the heading date to that measured for Himalaya, giving a further hint about the already known positive interplay between the

photoperiod and the gibberellin in the regulation of flowering time, overcoming the negative effect of cold temperature on this process.

1.1. *Sdw1*/*Denso* locus is needed for a proper flowering in low temperature

The *denso* gene encodes the *HvGA20ox2* enzyme, involved in the biosynthesis of gibberellin, and mutant plants in this gene show dwarfism and a delay in flowering time in normal temperature growth conditions (Jia *et al.*, 2009, 2011, 2015; Kuczyńska *et al.*, 2013, Xu *et al.*, 2017). To examine the effect of a mutation in the *denso* gene in cold temperature on the heading date, and its possible interplay with *VRN1*, I grew four double haploid lines which carry different alleles combination between *VRN1* and *denso* in low temperature. The chosen lines come from a doubled haploid population derived from the cross between SBCC145, which is a vernalization requiring variety, and Beatrix, which is a spring variety (Ponce-Molina *et al.*, 2012). The lines I used differ in the *denso* gene, and in the *VRN1* allele.

The doubled haploid lines DH-15 and DH-120 behave like spring lines, they do not require vernalization in order to flower. The DH-15 line carries a mutant allele of the *denso* gene, whereas the DH-120 line carries a wild type allele. A comparison between these two double haploid lines highlights the effect of a mutation in the *denso* gene on the heading date. The DH15 line, with a mutated *denso* allele, shows a late flowering phenotype in comparison to DH-120 line. Therefore, it is possible to conclude that in cold temperature growth conditions plants must have a perfectly functional GA signaling pathway in order to ensure a proper timing of flowering.

In varieties with low vernalization requirement, the presence of a wild type or a mutated allele of the *denso* gene does not seem to have an effect on the flowering time. The DH-201 and DH-463 double haploid lines show the same flowering time, in spite of the fact that they carry, respectively, a wild type *denso* allele and a mutated *denso* allele. It can be possible that the initial expression level of *VRN1* is high enough to lead the plants to flower without vernalization, and to overcome the delay in flowering time due to the presence of a mutation in the *denso* gene. Nevertheless, this does not explain why the DH-201 and DH-463 lines flower earlier than DH-15 and later than DH-120, which are spring varieties and should have higher *VRN1* expression levels.
In consequence, it is necessary to focus on other allelic differences of these doubled haploid lines, having a look to some other genes involved in the control of the flowering time, such as *VRN3*. The *VRN3* allele coming from the SBCC145 parental line cause a early flowering phenotype (Ponce-Molina *et al.*, 2012), and it is carried by DH-120, DH-201, and DH-463, whereas the DH-15 line carries the Beatrix allele of *VRN3*. This is a further hint proving the fact that DH-15 shows a later flowering time phenotype, in comparison to DH-120. Moreover, the lack of difference in the

heading date between DH-201 and DH463, despite of the fact that they carry, respectively, a wild type *denso* allele and a mutated *denso* allele, can be due to the fact that both inherited the *VRN3* allele from SBCC145. *VRN3* allele from SBCC145 causes early flowering time and would explain, then, the delay in the heading date shown by DH-15 in comparison to DH-120. The heading date is further delayed by the presence of the mutated *denso* allele. Moreover, it can explain the same flowering time showed by DH-201 and DH-463, and their difference in the heading date, in comparison to DH-15 and DH-120. In conclusion, it can be supposed that the presence of *VRN3* from SBCC145 could overcome the *denso* mutation which would cause a delay in the heading date, also explaining the same flowering time found in DH201 and DH463. Additionally, the combination spring growth habit, wild type *denso* allele and *VRN3* allele from SBCC145 seems to confer an extremely early flowering time phenotype, as it happens in the DH-120 line.

2. Meristem development is susceptible to temperature and gibberellin

The wind-pollinated flowers of cereals are unique structures. The inflorescence is composed by spikelets having individual flowers, called florets. The reproductive growth in cereals starts with the differentiation of the inflorescence meristem into the spikelet meristem, which becomes, afterwards, the floret meristem. Temperature, photoperiod, and vernalization affect the timing of flowering, influencing the shoot apical meristem development. In particular, chilling at floral initiation or during the reproductive phase suppresses the spikelet development or increases the spikelet sterility. Therefore, the microscopy analysis of the barley meristem development is a useful method to understand how cold and applied gibberellin can affect the growth of this structure and, eventually, to connect the meristem progression rate with the time to flower.

In normal temperature growth conditions (15°C), at 11 days after germination, the Golden Promise mock-treated seedlings have a meristem in the transition apex stage (Waddington stage 1), meaning that they are at the end of the vegetative phase. A comparison to *VRN1-HA(+)* transgenic lines shows that in Golden Promise the meristem development is slower. At 11 days after germination the meristem of *VRN1-HA(+)* seedlings is in the triple mound stage (Waddington stage 2.25). The difference in the developmental stage between Golden Promise and *VRN1-HA(+)* is probably due to the fact that, in the transgenic lines, the expression level of *VRN1* is higher than in Golden Promise. Moreover, the faster meristem development in *VRN1-HA(+)* lines reflects the accelerated heading date of these plants, in comparison to Golden Promise.

Applied GA has a very slight effect on the meristem development in Golden Promise seedlings at 10 and 11 days after germination. This slight effect is in line with the flowering time experiments, with which I could show that in Golden Promise the heading date is slightly accelerated by GA treatments.

I can conclude that exogenous applications of gibberellin accelerate the meristem development and the heading date in Golden Promise with the same rate. However, the accelerated meristem development in *VRN1-HA(+)* plants in response to applied GA does not reflect the flowering time experiment data. In those experiments, I could show that the transgenic lines do not further accelerate their flowering upon GA treatments, perhaps for a saturation of the GA responses. I can speculate that the apparent contrast between the faster meristem development and a non-response in the flowering time, can be due to the fact that the meristems are structure more sensitive to applied GA than the whole plant. Moreover, even if the meristems and the spikes, which will arise from, can develop faster upon GA treatment, the whole plant is not enough developed to sustain, from a nutritional point of view, the subsequent seed development. Indeed, the transgenic lines are smaller than the wild type plants and the number of fully developed seeds is lower (data not shown).
In conclusion, applied GA stimulates a non-synchronous and a non-proportional development in between meristem/spike and whole plant formation, which reflect the non-responsiveness of these transgenic lines to GA in terms of heading date.

The meristem developmental stages in mock-treated Himalaya seedlings are very similar to those found in Golden Promise, in both cultivars the meristems are still in the vegetative phase. Moreover, even in GA_3-treated seedlings, there are no differences in the meristem development between Himalaya and Golden Promise. It is possible to observe that the developmental stage seems to be slightly sensitive to gibberellin, in spite of the fact that in Himalaya the meristems are noticeably and significantly longer than in Golden Promise. This suggests that applied GA has a strong effect on their growth rate. At 11 days after germination, Himalaya meristems are in the early double ridge stage (Waddington stage 1.5), whereas in Golden Promise the meristems are still in the transition apex stage (Waddington stage 1). This result does not completely fit the data obtained with the flowering time experiments, which highlight that, in normal temperature growth conditions, Himalaya plants show a very early flowering time phenotype in comparison to Golden Promise plants. Then, it is possible to suppose that, despite of the fact that Himalaya seedlings grow faster than Golden Promise, and that the overall plant development is faster in Himalaya (data not shown), the meristem development in Himalaya seedlings is delayed during the early developmental stages.
The mock-treated *grd5* seedling mutants show a meristem development very similar to Himalaya, but a bit slower, probably due to the fact that the mutant line is impaired in the GA signaling pathway. By contrast, the *grd5* meristems are more sensitive to applied GA than Himalaya. At 11 days after germination the meristem of the mutant lines reached the double ridge stage (Waddington stage 2), whereas Himalaya meristems are in the early double ridge stage (Waddington stage 1.5). This result can be explained with the fact that, having an impaired GA

pathway, the *grd5* mutant line is more sensitive to GA treatment. However, the heading date is not significantly affected by exogenous GA applications, suggesting that probably the meristem growth in the *grd5* mutant is delayed during the later stages of development.

Low temperature growth conditions affect the meristem development of all the genotype analyzed. A comparison between mock-treated seedlings grown at 15°C and at 8°C shows that at 11 days after germination the meristems are in the vegetative phase and that during the days analyzed, 8, 9, and 10 days after germination, the growth rate of the meristems is significantly slower. The exception is the *VRN1-HA(+)* transgenic line, whose meristem grows faster and is in the early double ridge stage (Waddington stage 1.5) at 11 days after germination. The delay in the meristem development observed in low temperature reflects the delay in the heading date showed by plants grown in cold conditions. Moreover, the similarity in the meristem development between Himalaya and Golden Promise does not reflect the faster heading date shown by Himalaya plants. This suggests that, since meristems are very fragile structures and more sensitive to low temperature damage, their development is delayed irrespective to the cultivar during the earlier developmental stages.

Gibberellin applications in cold temperature seem to not have any effect on the meristem development in Golden Promise seedlings, at least between 8 and 11 days after germination. Apparently, this result is in contrast with the flowering time experiment, with which I could show that the time to heading of Golden Promise plants is significantly accelerated in cold temperature upon GA treatment. This difference suggests that so early in the development, the meristems are not sensitive to applied GA, and only during later stages of growth the meristems become more sensitive and accelerate their development, explaining why Golden Promise plants flower earlier upon GA treatments without observing any differences in the shoot apical meristem growth. By contrast, the *VRN1-HA(+)* transgenic seedlings show meristems slightly responsive to applied GA; at 11 days after germination the mock- and the GA_3-treated seedlings are, respectively, in the early double ridge stage and in the double ridge stage (Waddington stages 1.5 and 2). The weak responsiveness of *VRN1-HA(+)* meristems is in line with the fact that the transgenic lines do not accelerate their heading date upon GA treatment and, moreover, their accelerate meristem development reflects the fact that the *VRN1-HA(+)* lines flower earlier than Golden Promise plants.

Himalaya and *grd5* seedlings show meristems not very responsive to exogenous application of gibberellin, although the overall plant development is faster (data not shown). However, in the case of Himalaya, this is in line with the flowering time experiments, which show that this cultivar is not responsive to GA application. However, the meristem development in Himalaya and Golden Promise is very similar in mock- and GA_3-treated seedlings, and this does not reflect the faster

flowering time of Himalaya plants. It can be possible to speculate that, since the plants are growing in low temperature and long days conditions (16 hours light/8 hours dark) and Himalaya is sensitive to photoperiod, which is perceived from the leaves, the long day signal is used by the plants to accelerate the overall development in order to later sustain the growth of the meristems. In the case of *grd5* mutants, the non-responsiveness of their meristems does not reflect the fact that the mutant lines show an accelerated flowering upon GA applications. This result suggests that, even if the GA-treated plants grow faster, they are not able to sustain a proper meristem development and, therefore, there is a delay in the early stages of meristem development.

3. *VRN1* expression is affected by low temperature and gibberellin

In cereals, *VRN1* is well known for its key role in promoting the transition from vegetative to reproductive phase. Moreover, in barley and wheat, the activation of *VRN3* (*HvFT1*) is under the control of long days -through the activation by *HvCO* and *Ppd-H1*- and *VRN1* (Andrés and Coupland, 2012).
For this reason, in absence of other known mechanisms, it is possible to speculate that, in normal growth temperature conditions, in Golden Promise, *VRN3* is activated only by *HvCO* and *VRN1*, carrying, these plants, an inactive allele of *Ppd-H1* and being, therefore, insensitive to photoperiod. Consequently, in spite of the fact that *VRN3* has an important role in the flowering time control, in Golden Promise, its activation mostly depends on *VRN1* and then these plants flower later in comparison to Himalaya. Moreover, in Himalaya cultivar, which have an active *Ppd-H1* allele, long days and *VRN1* together contribute to a faster activation of *VRN3* and, in consequence, it is possible to observe an acceleration of flowering in these plants, even if their expression level of *VRN1* is slightly lower than those detected in Golden Promise.
These results clearly highlight the strong role of an active *Ppd-H1* allele in the regulation of flowering time, which almost seems to overcome the effect of *VRN1*.

Conversely, it is possible to observe a difference in *VRN1* expression when Himalaya and Golden Promise plants are treated with gibberellin. In Himalaya, following the treatment, *VRN1* transcription is inhibited, whereas in Golden Promise is enhanced. Despite the fact that this result seems to be inclined to an opposite direction, Himalaya plants show an early flowering phenotype in comparison to Golden Promise. This is a further and clear hint supporting the strong impact of an active *Ppd-H1* allele in the flowering time control in barley that can overcome, in Himalaya, the downregulation effect of gibberellin on *VRN1* levels and, thus, strongly accelerate the flowering.
In *VRN1-HA(+)* transgenic lines, the expression level of *VRN1* is higher in comparison to Golden Promise, explaining the fact that an high basal expression level of this gene is enough to strongly accelerate the flowering time. Moreover, the heading date of *VRN1-HA(+)* lines and Himalaya

plants is very similar, suggesting that high levels of *VRN1* can overcome the photoperiod insensitivity due to an inactive *Ppd-H1* allele of the transgenic lines. However, applied GA does not affect *VRN1* transcript level, suggesting that in these transgenic lines the gibberellin signaling pathway is saturated, and that *VRN1* already reached its maximum expression level.
The *grd5* mutant plants, which have an impaired GA biosynthesis pathway, show very low *VRN1* expression level, explaining the fact that these plants flower later than Himalaya plants, and suggesting that a proper function gibberellin biosynthesis pathway is required to ensure an adequate expression of *VRN1*, needed for a correct timing of flowering. However, even if *VRN1* levels in *grd5* plant are lower than Golden Promise, their heading date is comparable, probably due to a compensatory effect of the presence of an active *Ppd-H1* allele. Moreover, GA_3-treated *grd5* plants show a positive effect on *VRN1* transcript level, which do not correlate with the non-responsiveness of the flowering time of these plants probably because, even if *VRN1* is upregulated, its level is not enough to accelerate the flowering.

In low temperature conditions, the trend of *VRN1* level in Golden Promise as well as in *VRN1-HA(+)* transgenic lines, reflects the data obtained with the flowering time experiments. At 8°C, in comparison to 15°C, the stronger reduction of the heading date in Golden Promise correlates with the stronger upregulation of *VRN1*. According to my model, in low temperatures the catabolism of gibberellin is enhanced, reducing the amount of endogenous GA and, in Golden Promise, this leads to have a delay in the heading date. This delay can be partially rescued by GA treatments, which have a positive effect on *VRN1* expression level. The rescue of the heading date is only partial because, even if GA_3-treated Golden Promise plants flower earlier than mock-treated plants, the heading date at 8°C is still delayed in comparison to 15°C.
This result suggests that in Golden Promise, *VRN1* is positively regulated by applied GA and that the degree of its upregulation is dependent on the temperature to which the plant is subjected.
Similarly, in *VRN1-HA(+)* plants, the lack of difference in *VRN1* expression between mock and GA treatments, in both temperatures, is in line with the non-responsiveness of these plants to applied GA in terms of reduction of the heading date. Even in low temperatures, the lack of response of *VRN1-HA(+)* plants to applied GA is probably due to the fact that the GA signaling pathway is saturated, and that the endogenous amount of VRN1 is enough to ensure the promotion and the acceleration of flowering, overcoming the presence of an inactive *Ppd-H1* allele.

In Himalaya plants the endogenous *VRN1* level is lower in comparison to Golden Promise and *VRN1-HA(+)*, and the effect of applied GA on *VRN1* transcript level is limited in time. Himalaya plants flower earlier than Golden Promise, and the heading date is similar to *VRN1-HA(+)*.
This result strongly suggests that an active *Ppd-H1* allele and high endogenous *VRN1* level have a strong impact on promoting flowering, and a combination of one of these two factors with a

functioning GA signaling has an even stronger effect. In fact, in *grd5* mutants, impaired in the GA biosynthesis pathway but sensitive to photoperiod, the heading date is similar to Golden Promise and GA applications, which significantly upregulate *VRN1*, significantly accelerate the flowering.

As illustrated in Table 1, there is an inverse correlation between heading date, appearance of the double ridge structure, and *VRN1* expression levels. This correlation depends on the genotype of the plant, and it is affected by temperature and gibberellin treatment.

Table 1. Relationship between heading date, appearance of the double ridge structure, and *VRN1* expression levels.

	Golden Promise (genotype *HvVRN1-1*, *ΔHvVRN2*, *ppd-h1*)				***VRN1-HA(+)*** (as Golden Promise, with VRN1-HA construct)			
	15°C		**8°C**		**15°C**		**8°C**	
	- GA	**+ GA**	**- GA**	**+ GA**	**- GA**	**+ GA**	**- GA**	**+ GA**
Heading date (average days)	67.9	58.78 (*)	117.5	100.5 (****)	39.50	35.86 (n.s.)	81.3	77.3 (n.s.)
Appearance of the double ridge structure	n.d.	n.d.	n.d.	n.d.	9 dag	8 dag	10 dag	9 dag
VRN1 expression at 6 hours	0.093	0.137 (*)	0.435	1.000 (****)	2.424	2.417 (n.s.)	4.672	4.386 (n.s.)

	Himalaya (genotype *HvVRN1-1*, *ΔHvVRN2*, *Ppd-H1*)				***grd5*** (as Himalaya, with impaired GA signaling pathway)			
	15°C		**8°C**		**15°C**		**8°C**	
	- GA	**+ GA**	**- GA**	**+ GA**	**- GA**	**+ GA**	**- GA**	**+ GA**
Heading date (average days)	37.00	36.38 (n.s.)	76.0	70.5 (n.s.)	64.33	59.14 (n.s.)	117.7	61.0 (****)
Appearance of the double ridge structure	n.d.	11 dag	n.d.	n.d.	n.d.	10 dag	n.d.	n.d.
VRN1 expression at 6 hours	0.085	0.039 (*)	0.090	0.076 (*)	0.016	0.025 (*)	0.112	0.177 (***)

dag, days after germination; n.d., not detected. Asterisks indicate *P*-values of Student's *t*-test. (*P<0.05; ***P<0.001; ****P<0.0001; n.s., not significant).

4. Low temperature affects GA biosynthesis gene expression

The analysis of the variation of the genes involved in the gibberellin biosynthesis and deactivation pathway show that in barley these genes are subjected to temperature regulation. In cold temperature conditions the general trend of the GA biosynthesis genes, in all the genotype analyzed, suggests a balance between precursor production and GA activation. This could be, for the plant, a way to optimize the resources, limiting the production of the precursors and maximizing the production of bioactive GA. Moreover, it is possible to observe a cold-stimulated upregulation of one out of the three *HvGA2ox* genes tested - the *HvGA2ox* genes metabolize the active GAs and their precursors to inactive products - and this is consistent with the idea of the presence of a

feed-forward regulation, which would serve to stabilize and control the endogenous gibberellin concentration during plant growth, in order to keep active the GA developmental responses. In conclusion, in barley there is the evidence of a feedback mechanism that would maintain the endogenous concentration of bioactive GA within certain limits.

The analysis also highlights differences in the GA biosynthesis genes expression levels between Golden Promise and *VRN1-HA(+)* transgenic line, both in cold and in normal temperature growth conditions. The elevated amount of *VRN1* in the transgenic line is likely the cause of these differences. In fact, *Deng et al.*, 2015 conducted a transcriptome sequencing (RNA-seq) analysis and they found an elevated number of differentially expressed transcripts between *VRN1-HA(+)* and a sibling null line descended from the same transformation event. Among these, they found a putative gibberellin 2-beta-oxydase, more than one putative GID1 receptor, and a putative gibberellin 20 oxidase. These findings, together with the data show in this study, strongly suggest that a high *VRN1* expression level can affect the expression levels of the GA biosynthesis genes. The differences in their expression, together with the elevated amount of *VRN1* levels in the VRN1-HA(+) lines, are a further hint to explain the earlier flowering time phenotype as well as the faster meristem development of the transgenic line in comparison to Golden Promise

The analysis also shows differences between Himalaya and Golden Promise. In normal temperature growth conditions, the differences in the expression levels of the GA biosynthesis genes between Himalaya and Golden Promise highlight the effect of the presence of an active *Ppd-H1* allele. Furthermore, I could show that Himalaya plants flower earlier than Golden Promise, suggesting that an active *Ppd-H1* allele promotes flowering, likely increasing the GA content in Himalaya. Long day conditions upregulate in a significant manner *HvGA20ox1* and *HvGA3ox1* in Himalaya, suggesting a consequent increase of the endogenous amount of gibberellin.
An upregulation of *LpGA20ox1*, and a consequent increased amount of gibberellin are related to an early flowering phenotype in *Lolium perenne* and *Lolium temulentum*, which are sensitive to photoperiod, carrying an active *Ppd-H1* allele (MacMillan *et al.*, 2005). Taken together, these results suggest that the early flowering phenotype shown by Himalaya plants is most likely related to the fact that this cultivar carries an active *Ppd-H1* allele, differently from Golden Promise. Taking in account the fact that plants insensitive to photoperiod show a late flowering phenotype (MacMillan *et al.*,2005; Turner *et al.*, 2005; Shaw *et al.*, 2013), and that the flowering time experiments conducted in this study show that Golden Promise plants are late flowering in comparison to Himalaya, it is possible to suppose that the differential expression of the genes involved in the gibberellin biosynthesis is due to the presence of an active *Ppd-H1* allele and can be the basis of the differential flowering time phenotype of Himalaya and Golden Promise.

Even in low temperature, the presence of an active *Ppd-H1* allele seems to have a role in the regulation of GA biosynthesis expression. The downregulation of *HvGA20ox* genes and *HvGA3ox2*, required for the production of GA precursors and of the bioactive GA, respectively, suggested a reduction of GA content and, thus, a delay in the heading date at low temperatures. This is in line with the flowering time experiments, in Himalaya plants the heading date was delayed when plants were grown at 8°C, in comparison to 15°C. Moreover, the role of an active *Ppd-H1* allele in the regulation of the flowering time is clear because, irrespective to temperature, Himalaya plants flowered earlier than Golden Promise. At 15°C this can be explained by the fact that long days, in Himalaya, upregulate genes encoding enzymes which, in the gibberellin biosynthesis pathway, have a role in the production of precursors as well as bioactive GA. Consequently, it can be supposed an increase in the content of endogenous GA in the plants, which promotes flowering.

In low temperatures is less clear the reason why Himalaya plants, subjected to a photoperiod-dependent regulation GA biosynthesis genes, flowered earlier than Golden Promise, when the qRT-PCR analysis suggest a decrease in the gibberellin content in low temperatures. This is probably due to the fact that, at least in *Arabidopsis*, photoperiod plays an important role in the activation of FT, through the regulation of the circadian clock, and GA are involved in the activation of the meristem identity genes. Then, it can be suggested that this control also take places in barley plants: in Himalaya plants, responsive to photoperiod, the circadian clock is active and it activates *HvFT1* (*VRN3*), together with its activation by VRN1 and, additionally, with the GA acting in promoting the flowering; whereas, in Golden Promise, the activation of *VRN3* by the circadian clock is off and depends only on *VRN1*.

The *grd5* mutant is in Himalaya background, therefore it responds to photoperiod but its gibberellin pathway is impaired: it accumulates *ent*-kaurenoic acid in developing seeds and it is a GA-responsive dwarf mutant (Helliwell *et al.*, 2001). The behavior of the genes involved in biosynthesis and catabolism of gibberellin is very similar between Himalaya and *grd5* in response to temperature, with the exception of the *HvGA3ox* genes. *HvGA3ox1* does not change its expression in Himalaya, whereas in *grd5* it is downregulated; by contrast, *HvGA3ox2* is downregulated in Himalaya, whereas it does not change its transcript level in *grd5*. Moreover, the expression levels of the GA biosynthesis genes checked is very different between Himalaya and *grd5*, and this can be due to the fact that the mutant line is impaired in the GA biosynthesis pathway. A comparison between the expression levels of the GA biosynthesis genes in *grd5* and Himalaya suggests that in *grd5* the endogenous gibberellin content is higher than Himalaya, however in normal temperature conditions, as well as in cold temperatures, the mutant line flowers later than its wild type counterpart. Nevertheless, the heading date of the *grd5* mutant line is comparable to Golden

Promise then, it might be that the accumulation of *ent*-kaurenoic acid delays the heading date in *grd5* mutants, highlighting the fact that for a proper time to flower is required a functional photoperiod response as well as functional GA pathway.

5. Low temperature and gibberellin applications affect GA biosynthesis gene expression

In *Arabidopsis*, low temperatures and applications of exogenous gibberellin seem to have a similar effect on the GA biosynthesis process, as they both promote the GA catabolism by upregulating the *GA2ox* genes, and downregulating the *GA20ox* and the *GA3ox* genes. This forward regulation is well studied in *Arabidopsis*, but it is still not clear is if this mechanism is conserved in crop species, and in barley. I already observed that, in long term, in low temperature conditions, *HvGA20ox*, *HvGA2ox4* and *HvGA2ox5* genes are downregulated, while *HvGA3ox* and *HvGA2ox3* genes are upregulated. Thus, the question I want to answer is whether exogenous GA applications have an effect on the transcript levels of these genes and to evaluate the speed of response and for how long the response of the plants to applied GA overcomes the low temperature effect.

Generally, in Golden Promise and *VRN1-HA(+)* seedlings, it seems to be an extremely rapid response to the presence of exogenous GAs, but then low temperature responses seem to prevail in the plant, as if they wanted to brake the one that seems to be the beginning of a very rapid development of the plant in response to GA, when environmental conditions are not adequate for growth. Apparently, the plant seems to have a rapid upregulation of almost all the genes involved in the biosynthesis of gibberellin that have been tested, to apparently compensate the downregulatory effect of cold on the transcript levels of these genes. The time course proceeding of the transcription level of the genes involved in the GA biosynthesis process in mock-treated plants, seems to suggest that the plants work in order to not reduce the amount of precursors and bioactive GAs below of the threshold needed to maintain adequate developmental responses to GA, as suggested by the comparison of transcript levels between 15°C and 8°C. The application of exogenous gibberellin seems to initially hide to the plant the fact that growth is taking place in temperature conditions that are not suitable for a proper development. Therefore, it is possible to observe, in response to GA, an early upregulation of *HvGA20ox* genes and a subsequent decrease in their transcript levels. Similarly, *HvGA3ox2*, *HvGA2ox3* and *HvGA2ox4* genes respond, while *HvGA2ox5* show no responses to applied GA in cold. *HvGA3ox1*, conversely, shows a strong downregulation 6 hours after treatment.
Thus, it is possible to speculate that in Golden Promise as well as in *VRN1-HA(+)*, the general initial behavior seems to point in the direction of producing bioactive GAs and promote growth, although external conditions are not adequate. Later in time, however, unfavorable environmental

conditions of development appears to prevail or, in any case, appears to overcome the rapid initial response to gibberellin, and the plant seems to adjust the transcript levels in a way to maintain bioactive GAs in a quantity needed to have a proper development but, at the same time, to delay it.

In Himalaya plants it is possible to observe that, in cold temperature and upon GA_3 treatment, the genes involved in the biosynthesis of gibberellin respond quite rapidly. Generally, the *HvGA20ox* genes are downregulated on the long term. *HvGA2ox3* and *HvGA2ox4* are upregulated on the short term and, 6 hours after the treatment, their expression levels are subjected to a strong decrease; while *HvGA2ox5* is constantly upregulated. *HvGA3ox1* is also constantly upregulated, whereas *HvGA3ox2* show an initial upregulation followed by a downregulation 6 hours after the treatment. The trend of the *HvGA20ox* genes and the *HvGA2ox* genes upon GA_3 treatment appears to be a sort of compensatory behavior: an early upregulation followed by a downregulation, in a feedback mechanism implemented to control the production levels of the precursors of the bioactive forms of gibberellin. Moreover, the upregulation of *HvGA3ox1* and the downregulation of *HvGA3ox2* on the long term suggest a step further in the control of the endogenous amount of bioactive gibberellin.

6. GA biosynthesis gene expression changes rapidly in response to temperature changes

In order to ensure a proper growth and development, plants must be able to rapidly perceive changes happening in the surrounding environment and, likewise, they must be able to adapt to these changes modifying gene expression. Environmental temperature has a strong impact on plant growth and development; in consequence plants have to perceive temperature changes in order to not damage structures, such as meristems. Gibberellins are involved in the control of several developmental processes and their endogenous amount is finely regulated. This regulation implies the up or the down regulation of the genes involved in the synthesis and deactivation of gibberellins on the basis of changes in the surrounding environment as well as of the plant developmental stage. An adequate endogenous amount of gibberellin is required to keep active the GA-dependent growth and developmental responses. For these reasons I wanted to check how fast plants respond to changes in the environmental temperature, evaluating the changes in the expression of the genes involved in the GA synthesis and deactivation.

Changes in the environmental temperature are perceived very rapidly by barley seedlings. Reductions in temperature cause alterations in the expression of the genes involved in the GA synthesis and catabolism, which probably modify the amount of endogenous gibberellin, and also affect the plant development. In Golden Promise and *VRN1-HA(+)* transgenic lines, the *HvGA20ox*

genes are downregulated, suggesting a reduction in the production of the precursors of bioactive GA. Furthermore, *HvGA3ox1* is downregulated and *HvGA3ox2* is upregulated, indicating a decline in the production of bioactive GA. Additionally, there is a strong upregulation of the *HvGA2ox* genes, with the exception of *HvGA2ox3* which is downregulated in *VRN1-HA(+)*.

With slight differences, in Himalaya and *grd5 HvGA20ox1* is downregulated, *HvGA20ox2* does not change its expression levels in Himalaya, on the long term, whereas in *grd5* it is upregulated. *HvGA20ox4* has an opposite behavior, it is upregulated in Himalaya and downregulated in *grd5. HvGA2ox3* is upregulated. *HvGA2ox4*, *HvGA2ox5*, and *HvGA3ox2* are strongly upregulated, whereas *HvGA3ox1* is downregulated.

Taken together these results suggest that the total amount of bioactive GA is rapidly decreased in low temperatures, and support our model, which propose that cold temperature promotes the catabolism of gibberellin. Moreover, these data confirm the presence of a feedback loop mechanism implemented by the plant to finely control the production of bioactive GA, and that this feedback mechanism is activated very rapidly in response to changes in temperature.

Similarly, it is possible to observe a fast change in the expression of the genes involved in the synthesis of gibberellin when barley seedlings grown at 8°C are subjected to an increase of the temperature. In Golden Promise, *HvGA20ox1* decreases its expression, whereas in *VRN1-HA(+)* it is upregulated. *HvGA20ox2* is upregulated in Golden Promise and downregulated in the transgenic line. There is no change in the expression level of *HvGA20ox4* in Golden Promise; whereas in *VRN1-HA(+)* its expression is subjected to a strong and fast upregulation, followed by a strong downregulation until its initial level. *HvGA2ox3* is upregulated in both lines, but in Golden Promise, at 6 hours after the temperature alteration, it is downregulated to its initial level. *HvGA2ox4* and *HvGA2ox5* are both downregulated. *HvGA3ox1* and *HvGA3ox2* are downregulated in Golden Promise; whereas in the transgenic line, after an early and strong change in their expression, their levels are stabilized to the initial ones.

The increase of temperature has a similar effect on the expression of the GA biosynthesis genes in Himalaya and *grd5* mutant. *HvGA20ox1* is downregulated, *HvGA20ox2* and *HvGA20ox4* do not change their transcript levels, but *HvGA20ox4* shows an early and strong upregulation in Himalaya. *HvGA2ox3* is upregulated, whereas *HvGA2ox4* and *HvGA2ox5* are both downregulated, whereas *HvGA3ox1* and *HvGA3ox2* are both downregulated.

In all the genotype analyzed, observing the changing in the transcript levels of the GA biosynthesis genes, it seems that, apart from their rapid response, the endogenous amount of GA is not

affected or slightly decreases. These results suggest that, even if the plants respond quite rapidly to changes in temperature, the adjustment of the transcript levels of the genes involved in the synthesis and deactivation of gibberellin seems to not be enough to increase the endogenous amount if GA.

7. Concluding remarks

In summary, this thesis presents the temperature-dependent control of flowering time in barley by the GA signaling pathway, highlighting especially the interplay between VRN1 and the GA pathway in the context of cold temperature growth conditions, and demonstrating the temperature- and GA-dependent regulation of the genes involved in biosynthesis and deactivation of gibberellin in barley. The fact that the photoperiod pathway has a very strong impact in the regulation of flowering time, and that the role of SLN1 was not really unraveled, clearly demonstrate that much more work is needed to completely understand the complex mechanism of flowering time in cereals, and that the currently proposed model is too simplistic (Figure 26).

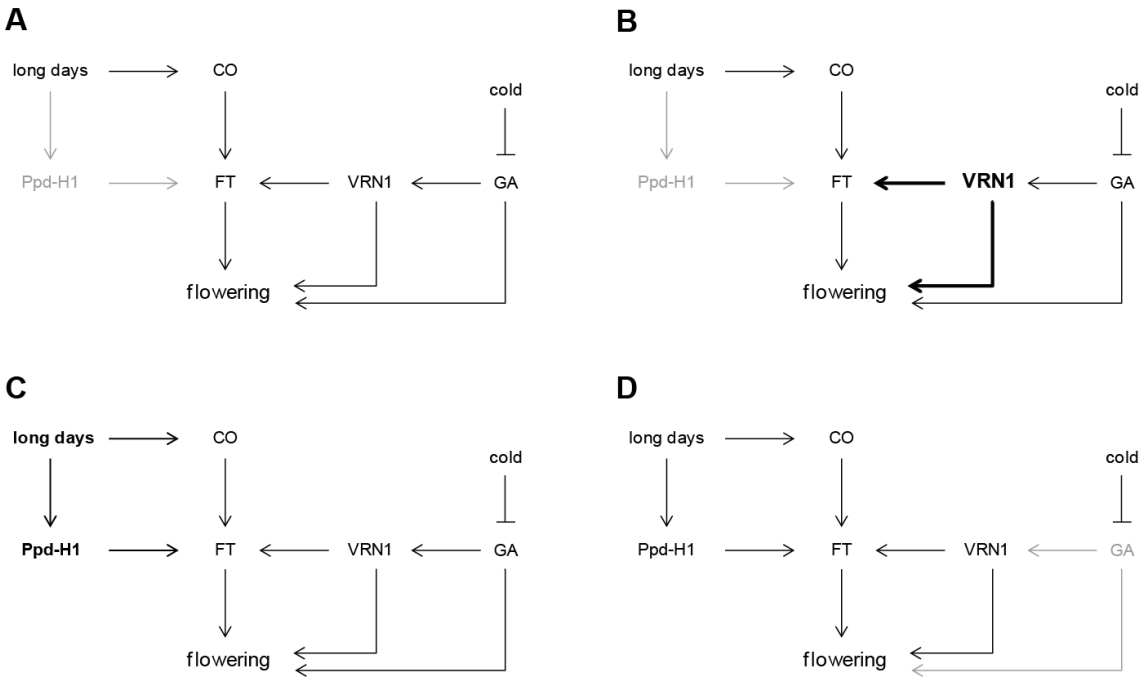

Figure 26. Model explaining the relation between VRN1 and GA signaling in the control of flowering time in barley. (A) in Golden Promise the photoperiod pathway is not active, and the flowering time mostly relies on the VRN1-dependent FT activation; (B) in *VRN1-HA(+)* the elevated *VRN1* expression is the basis of the early flowering time; (C) in Himalaya the photoperiod pathway has a very strong impact on flowering; (D)in *grd5* the GA pathway is impaired and flowering mostly relies on photoperiod-dependent activation. In grey, pathways inactivated or impaired; in bold (B) high VRN1 expression level, (C) prevalent role of photoperiod in flowering time control in Himalaya.

MATERIALS AND METHODS

1. Materials

1.1. *E. coli* strain

For plasmid propagations the *E. coli* strain DH5α was used [genotype: F^- Φ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F) U169 *rec*A1 *end*A1 *hsd*R17(r_k^-, m_k^+) *pho*A *sup*E44 *thi*-1 *gyr*A96 *rel*A1 λ]; for protein expression the *E. coli* strain BL21 was used [genotype: F–ompT hsdSB(rB–, mB–) gal dcm (DE3); (Studier and Moffatt, 1986)].

1.2. *S. cerevisiae* strain

The following *S. cereviasiae* strains were used for the yeast two hybrid assay: Y8800 [genotype: *MAT a leu2-3,112 trp1-901 his3Δ200 ura3-52 gal4Δ gal80Δ cyh2*^{R} *GAL2::ADE2*, *GAL1::HIS3-LYS2*, *GAL7::lacZ-met2* (Dreze *et al.*, 2010)], and Y8930 [genotype: *MAT α leu2-3,112 trp1-901 his3Δ200 ura3-52 gal4Δ gal80Δ cyh2*^{R} *GAL2-ADE2*, *GAL1::HIS3-LYS2*, *GAL7::lacZmet2* (Dreze *et al.*, 2010)].

1.3. Plant material

Plant materials of barley (*Hordeum vulgare* L.) used in this study included Golden Promise, a spring variety, which flowers without vernalization and is photoperiod insensitive [genotype *HvVRN1-1*, *ΔHvVRN2*, *ppd-h1*; (Deng *et al.*, 2015)]; and Himalaya, a spring variety which flowers without vernalization and is photoperiod sensitive [genotype *HvVRN1-1*, *ΔHvVRN2*, *Ppd-H1*; (Boden *et al.*, 2014)].

Transgenic plants used in this study are *VRN1-HA(+)*, in Golden Promise spring background [genotype *HvVRN1-1*, *ΔHvVRN2*, *ppd-h1*; (Deng *et al.*, 2015)], carrying the VRN1-HA transgene for the high basal expression level of *VRN1*, in comparison with Golden Promise plants (Deng *et al.*, 2015).

The *grd5* (M574) mutant is impaired in the gibberellin biosynthesis pathway. It carries a mutation in the *Grd5* (*GIBBERELLIN-RESPONSIVE DWARF5*) gene, which encodes a member of the CYP88A subfamily of cytochrome P450 enzymes. The developing grains accumulate *ent*-kaurenoic acid, and the plants are dwarf and responsive to applied GA (Helliwell *et al.*, 2001).

The doubled haploid lines DH-15, DH-120, DH-201, and DH-463 used come from a doubled haploid population derived from the cross between the Spanish landrace SBCC145 and the German variety Beatrix (Ponce-Molina *et al.*, 2012).

1.4. Antibodies

The following primary antibodies were used for the detection of proteins in the Western blot: αGST (1:2000; GE Healthcare Life Science); αHA-Peroxidase (1:1000; Roche, Penzberg, Deutschland). As secondary antibodies was used, αgoat-IgG-Peroxidase (1:1000; Sigma-Aldrich, St. Louis, USA).

1.5. Plasmids

1.5.1. Standard vectors

Table 2. List of cloning vectors used in this thesis.

Vector name	**Reference**
pGADT7 AD	Clontech Laboratories, Inc., Mountain View, USA
pGBKT7	Clontech Laboratories, Inc., Mountain View, USA
pGEX-4T-1	GE Healthcare Life Science, Little Chalfont, UK
pJET	Thermo Fisher Scientific, Waltham, USA

1.5.2. Constructs

Table 3. List of the constructs generated in this thesis.

Construct	**Vector**
VRN1-AD	pGADT7 AD
SLN1-BD	pGBKT7 BD
SLN1 ΔN-BD	pGBKT7 BD
SLN1 ΔN M5-BD	pGBKT7 BD
SLN1-GST	pGEX-4T-1

1.6. Primers

All primers were purchased from Sigma-Aldrich (St. Louis, USA).

Table 4. List of primers used for cloning.

Name	Sequence	Use	Notes
Hv28	gaattcATGGGGCGCGGGAAGGTGCA	*VRN1* Fw	EcoRI restriction site
Hv29	ggatccTCAGCCGTTGATGTGGCTCACCAT	*VRN1* Rev	BamHI restriction site
Hv48	ggatccGTTGTCGTAGACACTCAA	*SLN1* Fw	BamHI restriction site
Hv49	ctgcagTTACGGAGCAGCCAATCTCCA	*SLN1* Rev	PstI restriction site

Table 5. List of primers used for qRT-PCR.

Name	Sequence	Use	Notes
RT01	GGAAACTGAAGGCGAAGGTTGA	*VRN1* Fw	
RT02	TGGTTCTTCCTGGCTCTGATATGTT	*VRN1* Rev	
RT21	GCCGTGCTTTCCCTCTATG	*ACTIN* Fw	
RT22	GCTTCTCCTTGATGTCCCTTA	*ACTIN* Rev	
RT23	GGTACAAGAGCTGCCTCCAC	*HvGA20ox1* Fw	Boden *et al.*, (2014)
RT24	CACCACCTTGTCCATCTCG	*HvGA20ox1* Rev	Boden *et al.*, (2014)
RT25	CTACGAGCCAATGGGGAG	*HvGA20ox2* Fw	Boden *et al.*, (2014)
RT26	CCAGCAGCTCCATGATCCT	*HvGA20ox2* Rev	Boden *et al.*, (2014)
RT29	GGACGGCGAGGTGGAC	*HvGA20ox4* Fw	Boden *et al.*, (2014)
RT30	AAGGTGTCGCCGATGTTTAC	*HvGA20ox4* Rev	Boden *et al.*, (2014)
RT33	GCACTACCGCCACTTCTCTG	*HvGA3ox1* Fw	Boden *et al.*, (2014)
RT34	ACGAGGAACAGCTCCATCAG	*HvGA3ox1* Rev	Boden *et al.*, (2014)
RT35	GAAGCAGGTTTAACGCAAGA	*HvGA3ox2* Fw	Boden *et al.*, (2014)
RT36	TCTCTCTTCGGGGTCTCTTC	*HvGA3ox2* Rev	Boden *et al.*, (2014)
RT37	GCAGGTGCTGACCAACG	*HvGA2ox3* Fw	Boden *et al.*, (2014)
RT38	GGTGCAATCCTCTGTGTCAA	*HvGA2ox3* Rev	Boden *et al.*, (2014)
RT39	GACTCCCTCCAGGTTCTGAC	*HvGA2ox4* Fw	Boden *et al.*, (2014)
RT40	CGGCGAAGTAGATCATCG	*HvGA2ox4* Rev	Boden *et al.*, (2014)
RT41	CGAGGGTGTCCATGATCTTC	*HvGA2ox5* Fw	Boden *et al.*, (2014)
RT42	TTGTGGGTGCTGCTCTTGTA	*HvGA2ox5* Rev	Boden *et al.*, (2014)

1.7. Chemicals and Reagents

1.7.1. Chemicals

Table 6. List of chemicals used in this thesis

Name	Company
β-mercaptoethanol	Carl Roth, Karlsruhe, DE
2-Propanol	Carl Roth, Karlsruhe, DE
3-Amino-1,2,4-triazole (3-AT)	Sigma-Aldrich, St. Louis, USA
Acetic acid	Carl Roth, Karlsruhe, DE
Acrylamide	Carl Roth, Karlsruhe, DE
Adenin hemisulfate salt	Sigma-Aldrich, St. Louis, USA
Agar bacteriology grade	AppliChem, Darmstadt, DE
Agarose	PeqLab, Erlangen, DE
Albumine fraction V (pH 7,0) (BSA)	AppliChem, Darmstadt, DE
Ammonium peroxydisulphate [APS; $(NH_4)_2S_2O_8$)]	Carl Roth, Karlsruhe, DE
Ampicillin sodium salt	Carl Roth, Karlsruhe, DE
Brilliant Blue R-250	Carl Roth, Karlsruhe, DE
Bromophenol blue	Carl Roth, Karlsruhe, DE
Chloral hydrate ($C_2H_3Cl_3O_2$)	AppliChem, Darmstadt, DE
D(+)-Glucose ($C_6H_{12}O_6$)	Carl Roth, Karlsruhe, DE
D(+)-Saccharose ($C_{12}H_{22}O_{11}$)	Carl Roth, Karlsruhe, DE
Desoxyribonucleoside triphosphates (dNTPs)	Thermo Fischer Scientific
Dimethylsulfoxide (DMSO)	Carl Roth, Karlsruhe, DE
DNA salmon sperm sonified	AppliChem, Darmstadt, DE
Ethanol	CNL GmbH, Butzbach, DE
Ethylenediaminetetraacetic acid (EDTA)	Carl Roth, Karlsruhe, DE
Etidium Bromide	Carl Roth, Karlsruhe, DE
Gibberellic $acid_3$	Duchefa Biochemie, Haarlem, NL
Glycerol	Carl Roth, Karlsruhe, DE
Glycine	Carl Roth, Karlsruhe, DE
Hydrochloric acid fuming 37% (HCl)	Carl Roth, Karlsruhe, DE
Isopropyl β-D-1-thiogalactopyranoside (IPTG)	Carl Roth, Karlsruhe, DE
Kanamicin sulphate	Carl Roth, Karlsruhe, DE
L-gluthatione reduced	AppliChem, Darmstadt, DE
Lithium acetate ($C_2H_3LiO_2$)	AppliChem, Darmstadt, DE
Lithium chloride (LiCl)	Carl Roth, Karlsruhe, DE
L-leucine	Sigma-Aldrich, St. Louis, USA

Magnesium chloride ($MgCl_2$)	AppliChem, Darmstadt, DE
Methanol	Carl Roth, Karlsruhe, DE
Orange G	Carl Roth, Karlsruhe, DE
Phenol	Carl Roth, Karlsruhe, DE
Polyehtylene glycol 4000 (PEG4000)	Carl Roth, Karlsruhe, DE
Potassium acetate (KCH_3COO)	Carl Roth, Karlsruhe, DE
Potassium chloride (KCl)	Carl Roth, Karlsruhe, DE
Potassium dihydrogenphosphate (KH_2PO_4)	Carl Roth, Karlsruhe, DE
Powdered milk	Carl Roth, Karlsruhe, DE
Sodium acetate (NaOAc)	Carl Roth, Karlsruhe, DE
Sodium chloride (NaCl)	Carl Roth, Karlsruhe, DE
Sodium dodecyl sulfate (SDS)	Carl Roth, Karlsruhe, DE
Sodium hydrogen phosphate (Na_2HPO_4)	Carl Roth, Karlsruhe, DE
Sodium hydroxide (NaOH)	Carl Roth, Karlsruhe, DE
Super Signal West Femto Max. Sens. Substrate	Thermo Fischer Scientific, Waltham, USA
TEMED (Tetramethylethylenediamine)	Carl Roth, Karlsruhe, DE
Trichlormethan/Chloroform	Carl Roth, Karlsruhe, DE
Tris(hydroxymethil)-aminomethan (Tris)	Carl Roth, Karlsruhe, DE
Triton X-100	AppliChem, Darmstadt, DE
Tryptone/Pepton ex casein	Carl Roth, Karlsruhe, DE
Tryptophane	Sigma-Aldrich, St. Louis, USA
Tween 20	Carl Roth, Karlsruhe, DE
Uracil	Sigma-Aldrich, St. Louis, USA
Yeast extract	Carl Roth, Karlsruhe, DE
Yeast nitrogen base without amino acids	Sigma-Aldrich, St. Louis, USA
Yeast synthetic Drop-out medium supplement without Leucine and Tryptophan	Sigma-Aldrich, St. Louis, USA
Yeast synthetic Drop-out medium supplement without Leucine, Tryptophan, and Uracil	Sigma-Aldrich, St. Louis, USA
Yeast synthetic Drop-out medium supplement without, Histidine, Leucine, Tryptophan and Uracil	Sigma-Aldrich, St. Louis, USA

1.7.2. Enzymes and Kit

Table 7. List of enzymes and kits used in this thesis

Enzyme and Kit	Company
CloneJET PCR Cloning Kit	Thermo Fischer Scientific, Waltham, USA
DNase I, RNase-free	Thermo Fischer Scientific, Waltham, USA
iQ SYBR Green Super Mix	Bio-Rad Laboratories
M-MuLV Reverse Transcriptase	Thermo Fischer Scientific, Waltham, USA
Phusion High-Fidelity DNA Polymerase	Thermo Fischer Scientific, Waltham, USA
Recombinant RNasin® Ribonuclease Inhibitor	Promega, Madison, USA
Restriction enzymes	Thermo Fischer Scientific, Waltham, USA
RiboLock RNase Inhibitor	Thermo Fischer Scientific, Waltham, USA
RNase A	Carl Roth, Karlsruhe, DE
T4 DNA ligase	Thermo Fischer Scientific, Waltham, USA
TNT® T7/SP6 Coupled Wheat Germ Extract System	Promega, Madison, USA
Wizard® SV Gel and PCR Clean-Up System	Promega, Madison, USA

1.7.3. Markers

The markers used in this thesis were the Gene Ruler 1Kb DNA Ladder and the Page Ruler Prestained Protein Ladder (Thermo Fisher Scientific).

2. Methods

2.1. Plant growth conditions

All plants were grown in chambers at 8°C or 15°C with 60% humidity and light 200 µmol/m^2 sec, under long day conditions (16 h light/8 h dark).

2.2. Flowering time measurements

The heading date was determined as the day when the head first emerged from the sheath of the main stem.

2.3. Hormone treatments

Gibberellin treatments were performed using GA_3 (Sigma-Aldrich) prepared as stock solution (100 mM) in ethanol and diluted in water prior to application to a concentration of 0.1 mM. Treatments were applied to the plants by spraying twice per week until the end of the experiment.

2.4. Molecular biology methods

2.4.1. Polymerase chain reaction (PCR)

The polymerase chain reaction was used for the enzymatic *in vitro* amplification of a specific DNA segment of interest. For colony PCR, a standard Taq DNApolymerase was used; for cloning, the Phusion High-Fidelity DNA polymerase (Thermo Fisher Scientific) was used.

2.4.2. Agarose gel electrophoresis

DNA isolation and PCR products were subjected to 1% (w/v) agarose gels containing ethidium bromide to a final concentration of 0.5 µg/ml. Gels were cast and run in 1x TAE Buffer [40 mM Tris, 20 mM acetic acid, 1 mM EDTA]. Standard size marker was Gene Ruler 1kb DNA Ladder (Thermo Fischer Scientific). Prior to load onto the gel, the samples were mixed with 6x loading buffer [10 mM TrisHCl (pH 7.5), 0.15% (w/v) Orange G, 60% (w/v) glycerol, 60 mM EDTA].

2.4.3. DNA purification

For isolation and concentration of DNA fragments (50 bp up to 10 kb) out of PCRs, the Wizard® SV Gel and PCR Clean-Up System (Promega) was used. The purification was done according to the company's instructions.

2.4.4. Cloning with restriction enzymes

For the generation of the VRN1-AD construct, the VRN1 coding sequence was PCR amplified from cDNA with primers Hv28 and Hv29, and cloned into the EcoRI-BamHI site of the pGADT7 vector (Clontech). The SLN1 full length coding sequence was synthesized by Eurofins MWG Operon (Ebersberg), adding BamHI site at the ATG, and PstI and XhoI sites at the TGA to facilitate the cloning into BamHI-PstI site of the pGBKT7 vector (Clontech) and into BamHI-XhoI site of the pGEX-4T-1 vector (GE Healthcare Life Science). Moreover, into the synthesized sequence, a BglII silent site was create. The BglII site facilitated the generation of the SLN1 ΔN-BD construct, cutting SLN-BD with BamHI and BglII and subsequent plasmid recircularization. The SLN1 ΔN M5 was

PCR amplified with primers Hv48 and Hv49 using SLN1-BD as template, and cloned into the BamHI-PstI site of the pGBKT7 vector (Clontech)

2.4.5. Transformation of plasmids into *E. coli*

The transformation of plasmids into *E. coli* was done by heat shock. 10 ng plasmid were added to 100 µl of chemical competent *E. coli* cells. The sample was incubated on ice for 30 minutes. The heat shock was performed at 42°C for 1 minute, and then the cells were cooled down in ice for 1 minute. Afterwards 500 µl of LB media were added. The sample was incubated in a shaker at 37°C for 45 minutes. Afterwards the transformations were plated onto LB plates containing specific antibiotics. The plates were incubated overnight at 37°C.

2.4.6. Plasmid mini preparation from *E. coli*

The isolation of plasmid DNA from *E. coli* in small scale (mini preparation) was performed by alkaline lysis. For this, 3 ml LB media containing the construct specific antibiotic were inoculated with a single colony of *E. coli* containing the plasmid of desire and incubated overnight at 37°C and 180 rpm. The liquid culture was transferred into a 2 ml Eppendorf tube. The culture was centrifuged for 1 minute at 13200 rpm. The supernatant was completely removed and the pellet resuspended in 150 µl Buffer 1 [25 mM Tris-HCl (pH 8.0), 10 mM EDTA, 50 mM glucose]. Afterwards 150 µl freshly prepared Buffer 2 [0.2 M NaOH, 1% (w/v) SDS] was added and an incubation for 5 minutes at room temperature followed. Finally 150 µl Buffer 3 [3 M NaAc; 5 M acetic acid] was added and again the preparations were incubated on ice for 20 minutes. The sample was centrifuged for 10 minutes at 13200 rpm. The supernatant was transferred into a new Eppendorf tube, precipitated with 900 µl of 90 % ethanol and centrifuged for 10 minutes at 13200 rpm. The DNA pellet was washed with 70% ethanol and centrifuged for 5 minutes at 13200 rpm. The supernatant was completely removed and the pellet resuspended into 50 µl 1x TE with RNase [10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 5 µl/ml RNase]. The concentration and the purity of the isolated plasmid DNA was determined with a spectrophotometer (Thermo Fischer Scientific). The correctness of the inserted DNA sequence was checked by sequencing.

2.4.7. Isolation of total RNA

Total RNA was extracted using the lithium chloride (LiCl) extraction method (Verwoerd *et al.*, 1989), with slight modifications. Barley seedlings were collected in 15 ml tubes, frozen quickly in liquid nitrogen and stored at -80°C until use. The seedlings were ground using a mortar and a pestle (precooled in liquid nitrogen). After grinding, 500 µl RNA extraction buffer (10 mM Tris-HCl pH=8.0, 100 mM NaCl, 1 mM EDTA, 1% (w/v) SDS] and 500 µl of phenol:chloroform (1:1) were added. The mixtures were homogenized by vortex, and the samples were kept cooled in ice.

Afterwards, the samples were heated for 5 minutes at 60°C, homogenized by vortex each minute. After centrifugation for 10 minutes at 4°C at maximum speed, the upper phases were removed and mixed with one volume of chloroform. Then the samples were centrifuged again for 10 minutes at 4°C at maximum speed, the upper phases were removed, and mixed with one volume 8 M LiCI. RNAs were allowed to precipitate overnight in the cold room and collected by centrifugation. The pellets were dissolved in 300 µl sterile water, 0.1 volume of 3 M NaOAc and 2.5 volumes of 100% ethanol. Then the samples were centrifuged for 10 minutes at 4°C and the pellets were washed with 70% ethanol, dried and resuspended in 50 µl sterile water. RNAs concentration and purity were determined with a spectrophotometer (Thermo Fischer Scientific).

2.4.7.1. DNase I treatment

The DNase I treatment was performed to have DNA-free RNA. For the DNase I treatment, 1 µg of RNA was treated in a 10 µl batch with 10X reaction buffer with $MgCl_2$ and 1 U of RNase-free DNase I (Thermo Fisher Scientific). The reaction mixture was incubated for 30 minutes at 37°C, and then the reaction was stopped by adding 1 µl 50 mM EDTA and by incubating for 10 minutes at 65°C. The prepared RNA was used directly as template for reverse transcription.

2.4.7.2. cDNA synthesis

For the cDNA synthesis, 2 µg of RNA was synthesized in a 20 µl reaction with 2 mM oligo-dT primers (sequence: TTTTTTTTTTTTTTTTTTTTVN, T = thymine, V = adenine, cytosine or guanine, N = adenine, cytosine, guanine or thymine), 5X reaction buffer, dNTPs (250 µM dATP, 250 µM dCTP, 250 µM dGTP, 250 µM dTTP), 20 U of RiboLock RNase Inhibitor (Thermo Fischer Scientific) and 40 U M-MuLV Reverse Transcriptase (Thermo Fischer Scientific). The reaction mixture was incubated for 60 minutes at 37°C. The reaction was then stopped for 10 min at 70°C. The reverse transcription reaction was directly used in real time qRT-PCR.

2.4.8. Real time qRT-PCR (Real time quantitative reverse transcription PCR)

The expression levels of *HvGA20ox1* (MLOC_16059), *HvGA20ox2* (MLOC_56462), *HvGA20ox4* (MLOC_34543), *HvGA3ox1* (AY551430.1), *HvGA3ox2* (AY551431.1), *HvGA2ox3* (MLOC_38462), *HvGA2ox4* (AY551432.1), *HvGA2ox5* (AY551433.1) and *HvVRN1* (AY785826.1) genes were evaluated by real-time qRT-PCR, using iQ SYBR Green Supermix (Bio-Rad) in a 10 µl PCR assay. Primers are listed in Table 4. A 2-step program (Step 1: 50°C for 2 minutes; step 2: 95°C for 3 minutes; step 3: 95°C for 15 seconds; step 4: 60°C for 40 sec; step 2+3 repeated 39 times; step 5: 95°C for 10 seconds; step 5: 68°C to 95°C in 1°C steps in 5 seconds each) was performed with the CFX384 Real-Time System Cycler (Bio-Rad). For each experiment at least three biological replicates and three technical replicates were analyzed. Expression of candidate genes was

normalized against *HvACTIN* (AY145451.1). The experiments are presented as mean values and standard errors.

2.4.9. Yeast two hybrid assay

2.4.9.1. Small scale transformation of plasmids into *S. cerevisiae*

The small scale *S.cerevisiae* transformation was performed with the Lithium Acetate (LiAc)-mediated transformation from the *Yeast Protocols Handbook* (Clontech Laboratories). Yeast competent cells were prepared and suspended in a LiAc solution containing the appropriate plasmid DNA for transformation, and an excess of carrier DNA. Polyethylene glycol (PEG) with the appropriate amount of LiAc was added, and the mixture was incubated at 30°C. After the incubation, dimethyl sulfoxide (DMSO) was added, and the cells were heat shocked at 42°C, which allows the DNA to enter the cells. The cells were then plated in the appropriate medium to select the transformants containing the plasmid DNA. In yeast, selection is nutritional and the appropriate synthetic dropout (SD) medium was used.

2.4.9.2. Interaction analysis

To select the transformants containing the plasmid DNA, cells were plated on medium lacking Leucine or Tryptophane for the AD or the BD construct, respectively. Successful mating was tested using plates lacking Leucine and Tryptophane. The reporter genes of the yeast strains used in this thesis were *ADE2* and *HIS3*. To verify the interaction, cells were plated on medium lacking Leucine, Tryptophane and Histidine. To evaluate the strength of the interaction different concentrations of 3-Amino-1,2,4-triazole (3-AT) were added to the drop out media. The 3-AT compound is a competitive inhibitor of the product of the *HIS3* gene.

2.5. Biochemical methods

2.5.1. Protein expression in *E. coli* and GST purification

For the expression of GST-tagged proteins in BL21 cells, 4 ml of LB with antibiotics were inoculated with a single colony, and incubated overnight at 37°C in a shaking incubator. The next day, 500 ml of LB with antibiotics were inoculated with the precolture and the cells were grown for 3.5 to 4 hours at 37°C until the culture reached the OD_{600}: 0.5-1.0 After the addition of 0.5 mM isopropyl β-DThiogalactopyranoside (IPTG), cells were transferred to 30°C and incubated for 3 hours in a shaking incubator. The cells were harvested by centrifugation at 12,000 *g* for 10 min at 4°C and the pellet was resuspended in 1x PBS with protease inhibitor (139 mM NaCl, 2.7 mM KCl,

12.5 mM Na_2HPO_4, 1.8 mM KH_2PO_4) by vortexing. Then, the cells were sonicated 3 times for 1 minutes (50 cycles, 60% power). Afterwards, the proteins were solubilazed with Triton X-100 (final concentration 1%) and incubated with rotation 30 minutes at 4°C. The culture was centrifuged at 10,000 *g* for 10 minutes at 4°C to remove the pellet. To the supernatant was added 50-80 µl of Protino Glutahione Agarose 4B (Macherey-Nagel) in a ratio of 1:5 and it was incubated with rotation 30 minutes at 4°C. Then, the suspension was centrifuged for 3 minutes at 500 *g* at 4°C and washed 3 times with 1x PBS. After the last wash, 50 µl of 40 mM reduced gluthatione was added and the mixture was incubated with shaking at 4°C for 1-2 hours. Finally the mixture was centrifuged and the supernatant, containing the protein of interest, transferred in a new Eppendorf tube.

2.5.2. TnT® Coupled Wheat Germ Extract System

For the production of the VRN1-HA fusion protein, suitable for the GST and HA pull-down assays, the TnT® Coupled Wheat Germ Extract System was used according to the company's instructions.

2.5.3. *In vitro* pull-down assay

In the GST pull-down assay, 20 µl of Protino Glutahione Agarose 4B (Macherey-Nagel) were incubated with 50-80 pmol of SLN1-GST fusion protein and VRN1-HA fusion protein for 2 hours at 4°C with rotation. Afterward the beads were washed three times with 1x PBS (139 mM NaCl, 2.7 mM KCl, 12.5 mM Na_2HPO_4, 1.8 mM KH_2PO_4), resuspended in 2x Laemmli and heated at 95 °C for 5 min. The proteins were collected by centrifugation for 10 seconds at 500 g. Pulled-down proteins were analyzed by immunoblotting using αGST, and αHA-HRP antibodies. The HA pull down assay was performed as already described above for the GST pull-down assay, using Vector® Fusion-Aid™ – HA Kit (Vector laboratories, Burlingame, CA, USA).

2.5.4. SDS polyacrylamide gel

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed with Mini Protean II cells (Bio-Rad) as described in Laemmli (1970). For the preparation of the 5% stacking gel and 10-15% separation gel, acrylamide:bisacrylamide was used in a ratio of 29: 1.

2.5.5. Coomassie staining

SDS gels were incubated at room temperature for 1 hour with Coomassie staining solution (0.25% [w/v] Coomassie R-250, 50% ethanol, 10% acetic acid) on a shaker. Destaining solution (43% ethanol, 7% acetic acid) was used for destaining. The solution was changed several times and the gel was subsequently stored in water.

2.5.6. Western Blot

Proteins from SDS gels were exposed to a nitrocellulose blotting membrane (Amesham™ Protran™, GE Healthcare Life Science), using a semidry blotter. For transfer, semidry buffer [25 mM Tris-Base; 192 mM glycine; 20% (w/v) methanol; 1.3 mM SDS; pH 8.3] was used. For development via the peroxidase system, the membrane was incubated for 30 min in 5% milk powder in 1x PBS-T [1x PBS with 0.5% (w/v) Tween-20]. Incubation with the primary antibody was performed for 2 hours at room temperature or overnight at 4 ° C in 5% milk powder in 1x PBS-T. The membrane was washed three times in 1x PBS-T for 10 min and incubated with the secondary antibody for 2 hours at room temperature in 5% milk powder in 1x PBS-T. Then the membrane was washed again and the peroxidase activity was detected with the supersignal West Femto Maximum Sensitivity Substrate (Thermo Fischer Scientific) under the luminescence image Analyzer LAS-4000 miniseries (Fujifilm).

2.6. Microscopy

2.6.1. Sample preparation with the clearing method

Samples were harvested 8, 9, 10, 11 days after germination and fixed in a solution of ethanol:acetic acid (9:1), for 2 hours to overnight. The fixing solution was changed with 90% ethanol and the samples were incubated for 30 minutes to 1 hour at room temperature. Then, the 90% ethanol was changed with 70% ethanol. Afterwards, the ethanol was substituted with the clearing solution [3.869 M $C_2H_3Cl_3O_2$, 20% (w/v) glycerol]. The samples were mounted onto microscope slides and the microscope used for the meristem development analysis was OLYMPUS BX61.

2.7. Bioinformatics

2.7.1. Sequence analysis and database

The sequence analysis was done by Eurofins MWG Operon, Ebersberg; then the correctness of the sequences was checked using the free software ApE (A plasmid Editor). The sequence alignments were done with ClustalW2 (**{**HYPERLINK "http://www.ebi.ac.uk/Tools/msa/clustalw2/"**}**), Clustal Omega (**{**HYPERLINK "https://www.ebi.ac.uk/Tools/msa/clustalo/"**}**) and the free software ClustalX2. The database used to BLAST the sequences was the National Center for Biotechnology Information, NCBI (**{**HYPERLINK "https://www.ncbi.nlm.nih.gov/"**}**).

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