SQUINT promotes stem cell homeostasis and floral meristem termination in Arabidopsis through APETALA2 and CLAVATA signalling

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Abstract

Plant meristems harbour stem cells, which allow for the continuous production of new organs. Here, an analysis of the role of SQUINT (SQN) in stem cell dynamics in Arabidopsis is reported. A close examination of sqn mutants reveals defects that are very similar to that of weak clavata (clv) mutants, both in the flower meristem (increased number of floral organs, occasional delay in stem cell termination) and in the shoot apical meristem (meristem and central zone enlargement, occasional fasciation). sqn has a very mild effect in a clv mutant background, suggesting that SQN and the CLV genes act in the same genetic pathway. Accordingly, a loss-of-function allele of SQN strongly rescues the meristem abortion phenotype of plants that overexpress CLV3. Altogether, these data suggest that SQN is necessary for proper CLV signalling. SQN was shown to be required for normal accumulation of various miRNAs, including miR172. One of the targets of miR172, APETALA2 (AP2), antagonizes CLV signalling. The ap2-2 mutation strongly suppresses the meristem phenotypes of sqn, indicating that the effect of SQN on stem cell dynamics is largely, but not fully, mediated by the miR172/AP2 tandem. This study refines understanding of the intricate genetic networks that control both stem cell homeostasis and floral stem cell termination, two processes that are critical for the proper development and fertility of the plant.

Key words: APETALA2, CLAVATA signalling, floral meristem termination, flower development, SQUINT, stem cell homeostasis.

Introduction

Plant aerial growth results from the continuous production of new organs that derive from the shoot apical meristem (SAM), a dome of pluripotent cells, with a subpopulation of stem cells in the central zone (CZ). During vegetative growth, the SAM generates leaves, but, after the plant shifts to the reproductive phase, it is converted to an inflorescence meristem (IM) and generates flower meristems (FMs) on its flanks. Each FM in turn produces the floral organs that form a flower.

In Arabidopsis thaliana, stem cell maintenance in both the SAM and the FM relies on the homeodomain transcription
factor WUSCHEL (WUS; Laux et al., 1996). The expression domain of WUS underlies the stem cells (Mayer et al., 1998), but the WUS protein migrates to the stem cell niche, and this migration is necessary for stem cell maintenance (Yadav et al., 2011). Mutation of WUS causes premature SAM arrest, but does not prevent initiation of new meristems, which subsequently abort (Laux et al., 1996). Eventually, wus mutants produce some FMs, which similarly abort after producing a reduced number of floral organs.

In contrast, mutation in CLAVATA1 (CLV1), CLV2, CLV3, or CORYNE (CRN) causes a phenotype opposite to that of wus, with an increase in both SAM and FM size (Clark et al., 1993, 1995; Kayes and Clark, 1998; Muller et al., 2008). CLV1–CLV3 and CRN act in the same genetic pathway (Clark et al., 1995; Kayes and Clark, 1998; Muller et al., 2008). CLV1 encodes a receptor-like kinase with extracellular leucine-rich repeats (LRRs; Clark et al., 1997). CLV2 and CRN resemble CLV1, but CLV2 lacks the intracellular kinase domain (Jeong et al., 1999), while CRN lacks the extracellular LRRs (Muller et al., 2008). CLV1 forms homomers while CLV2 and CRN associate to generate a second receptor kinase complex (Muller et al., 2008; Bleckmann and Simon, 2009). CLV3 encodes a small, secreted protein that binds the CLV1 ectodomain (Fletcher et al., 1999; Rojo et al., 2002; Lenhard and Laux, 2003; Kondo et al., 2006; Ogawa et al., 2008; Ohyama et al., 2009). CLV3 might also bind CLV2, and signal simultaneously through the CLV1 and the CLV2–CRN receptor complexes (Muller et al., 2008). CLV3 also appears to signal in parallel through another receptor-like kinase, RPK2 (Kinoshita et al., 2010).

CLV signalling functions to restrict the number of cells that express WUS: the WUS expression domain expands in clv mutants, while WUS becomes undetectable when CLV3 is overexpressed (Brand et al., 2000; Schoof et al., 2000). In turn, WUS directly activates CLV3 expression in the stem cells (Yadav et al., 2011). Stem cell homeostasis in both the SAM and FM therefore relies on a negative feedback loop between WUS and the CLV pathway, but other genes contribute to the fine-tuning of the stem cell population. One example is APETALA2 (AP2), a transcriptional regulator that antagonizes CLV signalling (Wurschum et al., 2006).

However, the growth patterns of the SAM and FM exhibit a major difference. The SAM is indeterminate, in that it continuously produces new structures throughout the life of the plant, while the FM is determinate and generates a precise number of floral organs before stem cells cease to be maintained. Floral determinacy (also known as FM termination) relies on a flower-specific negative feedback loop superimposed on that of WUS/CLV. Together with LEAFY, WUS activates AGAMOUS (AG) in the centre of the stage 3 flower (Lenhard et al., 2001; Lohmann et al., 2001) stages as described by Smyth et al. (1990). AG, in turn, represses WUS both directly and indirectly, and WUS mRNA becomes undetectable by stage 6, when carpel primordia arise (Mayer et al., 1998; Sun et al., 2009; Liu et al., 2011). FM termination depends on proper AG expression in a subdomain at the centre of the flower, and numerous factors have been shown to be involved in AG transcriptional activation in this region (for reviews, see Prunet et al., 2009; Ito, 2011). clv mutants, which exhibit delayed stem cell termination in the FM, specifically lack AG expression in this central subdomain (Clark et al., 1993). This suggests that CLV signalling not only spatially restricts the population of stem cells within the FM, but also independently promotes their timely arrest through the activation of AG. Conversely, AP2 is a direct repressor of AG, and increased AP2 activity causes a loss of flower determinacy, associated with a defect of AG expression in the centre of the FM (Drews et al., 1991; Jofuku et al., 1994; Zhao et al., 2007; Yant et al., 2010).

Thus, defects in stem cell dynamics can result in two categories of phenotype. Defects in stem cell homeostasis cause changes in the size of the SAM and FM, and a variation in the number of floral organs within the four primary whorls. Conversely, a loss or delay of floral stem cell termination results in an indeterminacy phenotype, with extra whorls of floral organs forming within the flower. It is worth noting that both the CLV genes and AP2 affect both stem cell homeostasis and termination, although to different extents.

The role of SQUINT (SQN) in FM termination was previously characterized (Prunet et al., 2008). Mutation of SQN in a crabs claw (crc) mutant background causes a strong indeterminacy phenotype, with numerous supernumerary organs arising in between the carpels, a phenotype that results from a defect in AG expression in the centre of the FM (Prunet et al., 2008). SQN, which was initially identified for its role in vegetative phase change, encodes the Arabidopsis orthologue of cyclophilin 40, a putative chaperone (Berardini et al., 2001), and cannot regulate AG transcription directly. SQN was shown to be required for ARGONAUTE1 (AGO1) function and proper accumulation of miRNAs (Smith et al., 2009; Earley et al., 2010). SQN interacts with cytoplasmic Hsp90 proteins, which bind to AGO1 and trigger the formation of the mature RISC complex, and the interaction between SQN and Hsp90 is required for SQN function (Iki et al., 2010; Earley and Poethig, 2011). Accumulation of various miRNAs is reduced in sqn mutants (Smith et al., 2009), including miR172, a negative regulator of AP2 (Chen, 2004). This suggests a possible role for miR172 and AP2 in the sqn indeterminacy phenotype.

In this study, a detailed phenotypic analysis of a sqn allelic series demonstrates that sqn mutants have meristem phenotypes similar to that of weak clv mutants. Moreover, a sqn loss-of-function mutation has a very mild effect in a clv mutant background, but strongly suppresses the meristem abortion phenotype associated with the overexpression of CLV3. It is also demonstrated that a mutation in miR172d causes defects similar to those observed in sqn, and that the ap2-2 allele strongly, but not fully, suppresses the sqn meristem phenotypes. Taken together, the data suggest that SQN controls stem cell homeostasis and FM termination through the miR172/AP2 tandem and the CLV signalling pathway.

Materials and methods

Plant growth and crosses

Plants were grown in soil, first for 3 weeks under short-day (10 h light a day), 16 °C conditions, and then shifted to either long-day conditions, and then shifted to either long-day conditions and then shifted to either long-day conditions and then shifted to either long-day
(18 h light d−1), 20 °C or continuous day, 16 °C conditions. The strength of the meristem phenotypes of sqn and clv mutants varies with the growth conditions. This explains the discrepancies between different data sets presented herein. To eliminate this bias, each time mutants were compared, all genotypes were grown simultaneously with the growth conditions. This explains the discrepancies in growth rate (μ g l−1).

Transgenic plants were then generated as described by Bechtold and Pelletier (1998) and selected on Murashige and Skoog (MS) plates with kanamycin (50 μ g l−1−1).

**Results**

**Loss of SQN function results in flower defects similar to that of weak clv mutants**

Seven loss-of-function alleles of SQN have been described to date, all of which trigger a similar range of phenotypes, including defects in leaf initiation and shape, altered phyllotaxis, and increased carpel number (Fig. 1A, B, E; Berardini et al., 2001; Prunet et al., 2008; Smith et al., 2009). The floral phenotype of the sqn-1, sqn-4, and sqn-5 mutants, as well as two new T-DNA insertion alleles, sqn-8 and sqn-9, was characterized in detail. These mutants are all in a Col0 background, except for sqn-4, which is in the Ler ecotype. They display floral phenotypes that are similar but differ in strength. sqn-4 shows a significant increase in stamen and carpel number, but not in sepal and petal number (Fig. 1E). Conversely, sqn-1 and sqn-5 exhibit a significant increase in the number of all four types of floral organs (Fig. 1E). This increase in floral organ number is milder in the two outer whorls, and more severe in the two inner whorls. sqn-9 has a milder phenotype, with an increase only in stamen number (Fig. 1E). sqn-8 flowers do not differ from the wild type. In addition, stem cell termination is sometimes delayed in sqn-1 and sqn-5 flowers, with extra floral organs developing within the gynoecium (Fig. 1C; Table 1), a phenotype that was not observed in sqn-4, sqn-8, and sqn-9 when grown simultaneously for this experiment. However, the strength of the sqn phenotype varies depending on growth conditions, the general trend being that healthier, sturdier plants have a stronger phenotype. In different experiments, sqn-4 plants actually showed an enhanced phenotype, with indeterminate flowers (Fig. 1D, J). When the Col0 sqn alleles are ordered into an allelic series based on the severity of the flower phenotype, sqn-1 and sqn-5, which were inferred to be null alleles (Smith et al., 2009), are the strongest alleles, and sqn-8 and sqn-9 are the weakest alleles. With the notable exception of carpel number, the Ler allele sqn-4 appears intermediate, in terms of both floral organ numbers and indeterminacy. Yet, sqn-4 exhibits a stronger increase in carpel number than any other allele, including sqn-1 and sqn-5 (Fig. 1E), which may be an ecotype-specific effect, as the ERECTA gene was shown to control meristem size and WUS expression (Mandel et al., 2014). At the molecular level, sqn-1, sqn-4, and sqn-5 have point mutations or T-DNA insertions that generate early stop codons in the SQN mRNA, while sqn-8 and sqn-9 have T-DNA insertions in the SQN promoter (Supplementary Table S1 at JXB online). The SQN mRNA level appears reduced in sqn-1, sqn-4, and sqn-8, but, surprisingly, it is increased in sqn-5 and sqn-9 (Supplementary Fig. S1). However, the 3′ end of the SQN mRNA is missing in sqn-5 (Supplementary Fig. S1).

The flower phenotype of sqn-1, sqn-4, and sqn-5 mutant plants is similar to that of weak clv mutants such as clv1-2 and clv1-6, which show an increase in the number of all four types of floral organs, with a more pronounced increase in the inner whorls compared with the outer whorls (Fig. 1A, E; Clark et al., 1993). The extra carpels that develop in the fourth whorl of both sqn and clv mutant flowers sometimes arise in the upper half of the gynoecium, and modify its morphology.
**Fig. 1.** Floral phenotype of various mutant combinations. (A) From left to right, representative siliques of wild-type Col0 and Ler, sqn-1, sqn-4, sqn-5, sqn-8, and clv1-6 plants. (B) Left, silique of an sqn-4 plant showing an extreme increase in carpel number; right, representative silique of a clv3-2 plant. (C) Opened siliques of sqn-1 (left) and sqn-5 (right) plants grown in long-day conditions, showing extra carpels (red arrowheads) developing inside the gynoecium. (D) Opened silique of an sqn-4 plant grown in continuous-day conditions, showing extra carpels (red arrowheads) developing inside the gynoecium. (E) Floral organ numbers of wild-type (shades of grey), sqn single mutant (shades of blue), clv single mutant (shades of yellow), and sqn-4 clv double mutant (shades of purple) plants; a detailed key for the genotypes is on the right of the graph. Black and grey asterisks indicate significant differences from the wild type (Student’s t-test, P<0.01 and P<0.05, respectively). Red asterisks indicate significant differences from the corresponding single clv mutant (Student’s t-test, P<0.01). (F) Representative siliques of the clv1-4 (left) and sqn-4 clv1-4 (right) mutants. Red arrowheads point at the FM proliferating through the carpels, which are not fully fused at their tip. (G) Representative siliques of the clv3-2 (left) and sqn-4 clv3-2 (right) mutants. (H) Representative siliques of the crc-1 sqn-4 (left) and crc-8 miR172d-2 (right) mutants, showing a strong loss of FM determinacy. (I, J) Carpel number (I) and indeterminacy rate (J) of sqn-4 and sqn-4 ap2-2 mutants. The asterisk in I indicates a significant difference (Student’s t-test, P<0.0001). Scale bars=1mm in A, B, and H, and 500 μm in C, D, F, and G.
making it club-shaped (Supplementary Fig. S2 at JXB online; Clark et al., 1993; Prunet et al., 2008). Additionally, the gynoe-
cium of some sqn-4 flowers consists of up to eight carpels, similar to that of strong clv mutants such as clv1-4 and clv3-2 (Fig. 1B). Finally, sqn flowers have defects in stem cell termina-
tion, although to a lesser extent than clv flowers (Table 1; Clark et al., 1993, 1995; Kayes and Clark, 1998). Mutations in SQN and CLV1 also cause similar defects in other genetic back-
grounds. For instance, both sqn-4 ag-6 and sqn-4 ag-4 double mutant flowers are often fasciated; they appear much larger and contain many more floral organs than ag-6 and ag-4 single mutant flowers (Fig. 2, compare A and B, C and D), a pheno-
type also seen in clv1 ag double mutants (Clark et al., 1993).

sqn-4 exhibits enlarged inflorescence meristems and central zones
The expression of SQN is not restricted to flowers: SQN is broadly expressed throughout the plant (Prunet et al., 2008). Experiments were carried out to determine whether sqn also exhibits clv-like defects in the SAM. Inflorescences of sqn-4 mutant plants appear larger than those of wild-type plants (Fig. 3A, B), like those of clv mutants (Clark et al., 1993, 1995; Kayes and Clark, 1998). Live confocal imaging was used to assess the size of the SAM in wild-type and sqn-4 plants. The SAM of sqn-4 plants is significantly larger than that of the wild type, with a 55–92% increase in width, depending on the depth where the measurement is taken within the meristem (Fig. 3K). Also, the SAM of sqn-4 plants is sometimes fasciated (Fig. 3I, J; Supplementary Fig. S3 at JXB online), a phenotype that is typical of clv mutants and usually results in massively enlarged and flattened stems (Clark et al., 1993, 1995; Kayes and Clark, 1998). Interestingly, despite this occasional fasciation of the SAM, sqn-4 plants rarely exhibit flat stems. This may be due to the fact that branching of the meristem often follows fasciation in sqn-4 (Fig. 2J; Supplementary Fig. S3), thus preventing the formation of large, fasciated stems.

The size of the CZ was also assessed in wild-type and sqn-
umerances, using the pCLV3::erGFP reporter line (Reddy and Meyerowitz, 2005). The CZ appears both wider and higher in sqn-4 mutants compared with the wild type (Fig. 3C, D; Supplementary Fig. S4A, B at JXB online). In fasciated sqn-4

Table 1. Indeterminacy phenotype of sqn, clv, and sqn-4 clv mutants

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This table presents two independent data sets: an overall quantification of the indeterminacy phenotype of all mutants (left columns), and a more detailed analysis of this indeterminacy phenotype for some of these mutants (right columns).

n, number of flowers counted.

Fig. 2. sqn-4 causes fasciation in ag mutant flowers. (A) ag-6 flower. (B) sqn-4 ag-6 flower displaying many more floral organs than an ag-6 flower. (C) ag-4 flower. (D) Fasciated sqn-4 ag-4 flower. Scale bars=500 μm.
meristems, the CZ is linear, rather than circular (Fig. 3I, J), and often separates into two distinct domains (Fig. 3I), an event that probably precedes the branching of the meristem. Confocal z-stacks were processed with Imaris to quantify the volume of cells expressing \( pCLV3::erGFP \) within the SAM (Fig. 3G, H; Supplementary Fig. S4). The volume of the CZ is significantly increased in \( sqn-4 \) compared with the wild type (Fig. 3L). The \( CLV3 \) expression domain also appears enlarged in \( sqn-4 \) FMs (Fig. 3C, D), and, based on the level of GFP fluorescence, the \( CLV3 \) expression level is higher in \( sqn-4 \) flowers than in the wild type (Fig. 3E, F). Overall, this SAM and CZ enlargement phenotype reinforces the similarity between the \( sqn \) and \( clv \) mutants.

Genetic interactions suggest that \( SQN \) and the \( CLV \) genes act in the same pathway

The similarities between \( sqn \) and weak \( clv \) mutants prompted an investigation of the genetic interactions between \( SQN \) and the \( CLV \) genes. To this end, \( sqn-4 \) was crossed with one weak (\( clv1-6 \)) and two strong (\( clv1-4 \) and \( clv3-2 \)) \( clv \) alleles. \( clv1-6 \) results in a loss of \( CLV \) signalling through the \( CLV1 \) receptor complex only. In contrast, \( clv1-4 \) is a dominant-negative allele that is postulated to affect \( CLV \) signalling through both the \( CLV1 \) and \( CLV2–CRN \) receptor complexes, due to cross-talk between these complexes (Dievart et al., 2003; Muller et al., 2008). Signalling through both receptor complexes is similarly affected in \( clv3-2 \), due to the lack of functional ligand. \( CLV \) signalling is thus only partly defective in \( clv1-6 \), while it is strongly or fully impaired in \( clv1-4 \) and \( clv3-2 \).

Both \( clv1-4 \) and \( clv3-2 \) have enlarged SAMs and FMs, with an increase in primary floral organ number, and delayed stem cell termination in the FM (Fig. 1E–G; Table 1; Clark et al., 1993, 1995). After one or two extra whorls have been produced, the FM usually overproliferates, becomes irregularly shaped, and sometimes grows through the apex of the gynoecium, where carpels are often imperfectly fused (Fig. 1F).
sqn-4 clv1-4 and sqn-4 clv3-2 double mutants are morphologically indistinguishable from clv1-4 and clv3-2 single mutants, respectively (Fig. 1F, G), thus the double mutants had to be identified via PCR-based genotyping. However, some statistical differences were observed between the sqn-4 clv double mutants and the corresponding clv single mutants. Surprisingly, the number of sepals and carpels is slightly, but significantly reduced in sqn-4 clv1-4 compared with clv1-4, and the number of sepals and petals is similarly reduced in sqn-4 clv3-2 compared with clv3-2 (Fig. 1E). However, the main difference between sqn-4 clv double mutants and the corresponding clv single mutants lies in the strength of the indeterminacy phenotype: the loss of stem cell termination in clv1-4 and clv3-2 flowers is statistically increased by the sqn-4 mutation. Most clv1-4, clv3-2, sqn-4 clv1-4, and sqn-4 clv3-2 flowers are indeterminate (Table 1). Nevertheless, the proportion of sqn-4 clv1-4 flowers either producing six whorls (i.e. two extra whorls inside the gynoecium) or exhibiting an overgrowth of the FM through the primary carpels is higher than that of clv1-4 flowers (Table 1). Similarly, the proportion of sqn-4 clv3-2 flowers producing at least five or six whorls is higher than that of clv3-2 flowers (Table 1). Even though sqn subtly alters the clv phenotypes, the phenotype of sqn-4 clv1-4 and sqn-4 clv3-2 double mutant flowers always remains within the range of phenotypes caused by the clv1-4 and clv3-2 mutations alone, suggesting that SQN controls stem cell dynamics largely through the same pathway as the CLV genes.

The sqn-4 mutation has little effect on the primary floral organ number of the weak clv6-6 mutant (Fig. 1E), but it strongly modifies its indeterminacy phenotype. While only one-third of clv1-6 flowers exhibits a fifth whorl and none produces a sixth whorl, almost every sqn-4 clv1-6 flower produces a fifth whorl, and nearly one-third develops a sixth whorl (Table 1). Thus, sqn-4 enhances the loss of the FM indeterminacy phenotype of clv1-6, making it more similar to strong clv alleles, further supporting the idea that SQN and the CLV genes control stem cell dynamics through the same genetic pathway.

SQN is required for constitutive CLV signalling

In an effort to determine whether the sqn mutation affects constitutive CLV signalling, which normally results in a wus-like phenotype, with a premature arrest of both SAM and FMs (Brand et al., 2000), wild-type and sqn-4 plants were transformed with a 35S::CLV3 construct. All 35S::CLV3 plants that we analysed (n=14) initiated, but failed to maintain SAMs, resulting in a bushy and highly branched phenotype similar to that of wus mutants (Fig. 4B, C). Some 35S::CLV3 plants bolted (Fig. 4C), but their IMs aborted after producing a few terminal leaves or flowers (Fig. 4C, E, F). Conversely, nine out of 10 sqn-4 35S::CLV3 plants maintained their IMs (Fig. 4D, G-J), although the inflorescences were sometimes smaller than those of wild-type plants (Fig. 4G, K). A similar effect of the sqn-4 mutation was observed on flower development in plants overexpressing CLV3. Like wus mutants, 35S::CLV3 flowers exhibited a reduced number of stamens and no carpels, and, after senescence, no floral organs remained on the peduncle (Supplementary Fig. S5A at JXB online). Conversely, sqn-4 35S::CLV3 flowers usually produced a normal number of stamens and either a normal gynoecium (Fig. 4D, J) or a carpelloid filament, which remained on the peduncle after senescence of the flower (Fig. 4H, J; Supplementary Fig. S5B). Quantitative reverse transcription PCR (RT-PCR) analyses confirmed that these differences were not due to co-suppression of CLV3 in the sqn-4 mutant background, as 35S::CLV3 and sqn-4 35S::CLV3 plants expressed CLV3 at similar, very high levels (Fig. 4A). Moreover, quantification of WUS expression showed that overexpression of CLV3 was sufficient to reduce WUS expression strongly in an otherwise wild-type background, but failed to down-regulate WUS in sqn-4 consistently (Fig. 4A). Thus, the sqn-4 mutation strongly rescues both the IM and the FM phenotype of 35S::CLV3 plants, suggesting that SQN is required for constitutive CLV signalling.

ap2-2 strongly suppresses the meristem phenotypes of sqn

SQN is required for the proper accumulation of several miRNAs (Smith et al., 2009). The level of the miRNA miR172, in particular, is reduced by 40% in sqn-1 compared with the wild type. The targets of miR172 include AP2, which antagonizes both AG and the CLV pathway (Drews et al., 1991; Jofuku et al., 1994; Chen, 2004; Wurschum et al., 2006; Zhao et al., 2007). Thus, down-regulation of miR172 in a sqn mutant background could account for the meristem phenotypes described above. The potential role of miR172 and AP2 in the floral phenotype of sqn mutants was therefore investigated.

Unlike sqn mutants, neither the miR172a-1 and miR172d-2 single mutants nor the miR172a-1 miR172d-2 double mutant exhibit extra carpels in the fourth whorl or extra organs within the gynoecium, but miR172d-1 single mutant flowers were recently reported occasionally to have three carpels (Yumul et al., 2013). Similarly, the miR172a-1 mutation has no effect in a crc-8 mutant background. However, 9.6% of crc-8 miR172d-2 double mutant flowers (n=100) are indeterminate, and occasionally exhibit a strong loss of FM termination, with numerous stamens and carpels borne on a stem growing outside the primary gynoecium, a phenotype also seen in crc-1 sqn-4 flowers (Fig. 1H; Prunet et al., 2008). This phenotype is statistically stronger in the crc-8 miR172a-1 miR172d-2 triple mutant, with 17.7% of flowers being indeterminate (n=100). Despite this similarity, the indeterminacy phenotype of crc-8 miR172d-2 and crc-8 miR172a-1 miR172d-2 plants is statistically weaker than that of crc-1 sqn-4, in which 100% of flowers (n=100) are indeterminate. Nonetheless, the similar effect of the sqn-4 and miR172d-2 mutations in a crc mutant background, together with the increased carpel number in both sqn-4 and miR172d-1, suggests that the down-regulation of miR172 may be at least partly responsible for the meristem phenotype of the sqn mutants.

The role of one of the targets of miR172, AP2, was thus investigated in this phenotype. The ap2-2 allele strongly suppresses both the increase in carpel number and the
indeterminacy phenotype of *sqn-4* mutant flowers (Fig. 1I, J). However, this suppression is not complete, as some *sqn-4 ap2-2* flowers exhibit three or four carpels, and sometimes a fifth whorl, a phenotype never observed in *ap2-2*. Altogether, the data suggest that the meristem phenotypes of *sqn* mutants are largely, but not entirely, due to a decrease in miR172 levels and a subsequent increase in AP2 levels.

**Discussion**

*SQN* controls stem cell homeostasis by modulating CLV signalling downstream of CLV3

*SQN* was initially described for its role in promoting juvenile-to-adult phase change and FM termination (Berardini et al., 2001; Prunet et al., 2008). Here, it is shown that *SQN* also

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Fig. 4. The *sqn-4* mutation rescues the 35S::CLV3 phenotype. (A) From left to right: CLV3 mRNA levels in wild-type, 35S::CLV3, and *sqn-4 35S::CLV3* inflorescences, and WUS mRNA levels in wild-type, 35S::CLV3, and *sqn-4 35S::CLV3* inflorescences. CLV3 mRNA was below the detection threshold (BT) in the wild type. The CLV3 mRNA level does not significantly differ between 35S::CLV3 and *sqn-4 35S::CLV3* inflorescences. The WUS mRNA level significantly differs between 35S::CLV3 and *sqn-4 35S::CLV3* inflorescences (Student's t-test, *P* <0.01), but not between wild-type and *sqn-4 35S::CLV3* inflorescences. Error bars represent SEM. AU, arbitrary units. (B–D) Overall morphology of 35S::CLV3 (B, C) and *sqn-4 35S::CLV3* plants (D). (B) 35S::CLV3 plant that failed to bolt; regular initiation and subsequent abortion of SAMs confers on the rosette a bushy aspect. (C) 35S::CLV3 plant that eventually bolted; the IM aborted (white arrowhead) after producing two terminal leaves. (D) *sqn-4 35S::CLV3* plant that maintained several IMs, which produced normal siliques (red arrowheads) after bolting. (E–L) Apical view (E, G, I, K) and side view (F, H, J, L) of inflorescences of 35S::CLV3 (E, F), *sqn-4 35S::CLV3* (G, J), and wild-type plants (K, L). Red asterisks indicate carpelloid filaments; the red arrowhead points to a normal silique. Scale bars=1500 μm.
controls stem cell homeostasis in both the SAM and FM. Several pieces of evidence suggest that SQN acts through the same genetic pathway as the CLV genes. First, a detailed phenotypic analysis reveals that the sqn and clv mutants have similar phenotypes, both in the SAM, which appears enlarged and occasionally fasciated (Fig. 3), and in the FM, which produces more floral organs than the wild type, and, sometimes, extra whorls within the gynoecium (Fig. 1; Table 1; Clark et al., 1993, 1995; Kayes and Clark, 1998). In both sqn and clv mutants, these meristem phenotypes are associated with an increase in the stem cell population, as well as a prolonged maintenance of the stem cells in the flower, as indicated by the monitoring of the CLV3 stem cell marker (Fig. 3; Fletcher et al., 1999; Prunet et al., 2008). Mutations in SQN and the CLV genes also result in similar defects in an ag mutant background, with the formation of fasciated flowers (Fig. 2; Clark et al., 1993).

Secondly, an sqn loss-of-function mutation enhances the phenotype of a weak clv allele, but has only a mild effect in a strong clv mutant background. Despite some statistical differences, sqn clv double mutants are morphologically indistinguishable from clv single mutants. This is in stark contrast to what is observed when other mutants that affect meristem size, such as ultrapetala1 (ult1), are crossed to the clv mutants. Mutations in ULT1 and SQN cause very similar defects, including an increase in SAM size and floral organ number, a delay in FM termination, and a reduction of AG expression in the centre of the FM (Figs 1, 3; Fletcher, 2001; Carles et al., 2004; Prunet et al., 2008); yet, in contrast to sqn clv, the phenotype of ult1 clv double mutants is dramatically stronger than that of clv single mutants (Fletcher, 2001).

Thirdly, the sqn mutation strongly prevents the repression of WUS and the associated was-like phenotype normally caused by the overexpression of CLV3, showing that SQN is required for constitutive CLV signalling, and acts downstream of CLV3. Fourthly, WUS is epistatic to both SQN and the CLV genes (Schoof et al., 2000; Prunet et al., 2008). Finally, SHEPHERD (SHD), another chaperone that belongs to the Hsp90 family, was suggested to control stem cell homeostasis through the CLV pathway (Ishiguro et al., 2002). Like sqn, a mutation in SHD causes a clv-like phenotype, has little effect in a clv mutant background, and suppresses the was-like phenotype associated with the overexpression of CLV3. Altogether, these data indicate that SQN and the CLV genes largely act in the same genetic pathway to restrict WUS expression and control stem cell homeostasis. Based on this, SQN probably functions to promote CLV signalling downstream of CLV3 (Fig. 5).

The effect of SQN on CLV signalling appears sufficient to explain the increase in floral organ number and meristem size in the sqn mutants. It may also contribute to the delay in stem cell termination in sqn mutant flowers. The indeterminacy phenotype of sqn mutants was previously linked to a decrease in AG expression in the centre of the flower, but it was argued that this down-regulation was probably indirect, given the putative chaperone function of SQN (Prunet et al., 2008). A similar defect in AG expression has been described in the centre of clv mutant flowers (Clark et al., 1993), making the CLV signalling pathway one likely intermediate between SQN and AG (Fig. 5). However, while the sqn mutation has little effect on floral organ number in clv mutant flowers, it significantly enhances the indeterminacy phenotype of clv mutants (Table 1). This suggests that modulation of CLV signalling by SQN may fully account for the role of SQN in stem cell homeostasis, but not in floral meristem termination.

The miR172/AP2 tandem mediates an important part of the effects of SQN on stem cell dynamics

How does SQN, a putative chaperone, influence CLV signalling? While the Hsp90 protein SHD was proposed to
assist the folding of members of the CLV pathway (Ishiguro et al., 2002), available data suggest that SQN is unlikely to function via the same molecular mechanism. First, SQN is cytoplasmic, while SHD is localized in the endoplasmic reticulum (Ishiguro et al., 2002; Prunet et al., 2008). Secondly, no interaction between SQN and members of the CLV pathway was identified through yeast two-hybrid experiments (data not shown). Thirdly, both SQN and Hsp90 proteins were shown to promote AGO1 function (Smith et al., 2009; Earley et al., 2010; Iki et al., 2010). While no direct interaction between SQN and AGO1 has been shown, SQN binds to cytoplasmic Hsp90 proteins, which bind to AGO1, and the interaction between SQN and Hsp90 is required for SQN function (Smith et al., 2009; Earley et al., 2010; Earley and Poethig, 2011). Reduced AGO1 function in sqn causes a reduction in the accumulation of various miRNAs, which can potentially explain most of the phenotypes of the sqn mutant (Smith et al., 2009). Down-regulation of miR156 is critical for the precocious vegetative phase-change phenotype of the sqn mutant, and may also explain its aberrant phyllotaxis (Smith et al., 2009), but it is unlikely to account for the sqn meristem phenotypes. The accumulation of miR172, which targets AP2 (Aukerman and Sakai, 2003; Chen, 2004), is also strongly reduced in sqn (Smith et al., 2009). Since AP2 both represses AG and antagonizes CLV signalling (Drews et al., 1991; Jofuku et al., 1994; Wurschum et al., 2006), it seemed likely that reduced levels of miR172, and the resulting increase in AP2 protein abundance, cause, or at least contribute to, the meristem phenotypes observed in sqn mutants. Indeed, the fact that a mutation in miR172d triggers an increase in carpel number (Yumul et al., 2013), as well as an indeterminacy phenotype similar to that of sqn in a crc mutant background (Fig. 1H), supports this hypothesis. Accordingly, the strong ap2-2 allele strongly suppresses both the increase in carpel number and the floral indeterminacy phenotype of sqn mutants, suggesting that the miR172/AP2 tandem mediates an important part of the effect of SQN on both stem cell homeostasis and FM termination (Fig. 5).

The involvement of AP2 downstream of SQN probably explains why a mutation in SQN has little effect on floral organ number in clv mutants, but enhances flower indeterminacy. AP2 influences stem cell homeostasis by antagonizing CLV signalling (Wurschum et al., 2006; Zhao et al., 2007), but potentially affects FM termination through two different pathways: one independent of, and one dependent on, CLV signalling, both of which converge on AG (Fig. 5). AP2 represses AG directly, but also indirectly, through its effect on the CLV pathway, which promotes AG expression in the centre of the flower (Drews et al., 1991; Clark et al., 1993; Jofuku et al., 1994).

It is also worth noting that when Wurschum et al. (2006) proposed that AP2 affects CLV signalling, they could not rule out the alternative possibility that AP2 regulates WUS antagonistically, but independently of the CLV pathway. By linking SQN to the CLV pathway via AP2, the present data confirm that AP2 affects CLV signalling downstream of CLV3.

Other factors may contribute to the effect of SQN on stem cell homeostasis

The ap2-2 mutation does not completely suppress the increase in carpel number and floral indeterminacy of sqn mutants, suggesting that part of the effect of SQN on stem cell homeostasis and termination is independent of AP2. In addition to AP2, miR172 targets five other genes (Park et al., 2002; Aukerman and Sakai, 2003; Schmid et al., 2003; Chen, 2004). These six target genes function partially redundantly to control flowering time (Yant et al., 2010). This redundancy may similarly explain why the ap2-2 mutation fails to suppress the phenotype of sqn fully, as some of the five other targets of miR172 may compensate for the loss of AP2 and repress AG and CLV signalling in these mutants (Fig. 5).

It is also possible that miRNAs other than miR172 are involved (Fig. 5). For instance, miR165/166 and their HD-ZIPIII targets have been shown to affect stem cell homeostasis and termination (Prigge et al., 2005; Williams et al., 2005; Ji et al., 2011). The accumulation of miR165 is reduced by 10% in sqn-1 compared with the wild type (Smith et al., 2009), but the levels of miR166 have not yet been investigated. In any case, it is difficult to foresee the potential effects of decreased levels of miR165/166 on stem cell dynamics in the SAM and FM, as the influence of HD-ZIPIII genes on stem cell homeostasis is complex. Gain-of-function, miR165/166-resistant alleles of PHABULOSA (PHB) and PHAVOLUTA (PHV) cause flower indeterminacy in some mutant backgrounds, but do so overexpression of miR165/166 (Ji et al., 2011). Increased levels of miR166 in the jabhal-D mutant, and the subsequent decrease in PHB, PHV, and CORONA (CNA) expression, result in SAM fasciation but also in a reduction in carpel number (Williams et al., 2005). In contrast, the simultaneous loss of function of PHB, PHV, and CNA triggers an increase in carpel number and occasional indeterminacy (Prigge et al., 2005). The somewhat contradictory effect of HD-ZIPIII genes on stem cells suggests that reduced levels of miR165/166 may contribute to the AP2-independent meristem phenotypes of sqn (Fig. 5). Interestingly, this contradictory effect might also explain why the number of some floral organs is slightly reduced in some sqn clv double mutants compared with their clv single mutant counterparts (Fig. 1), a surprising result given that sqn mutants have more extra organs than the wild type, not less. To determine the proportional importance of miR172-dependent and independent mechanisms in the meristem phenotypes of sqn mutants would require the examination of a plant that is mutant for SQN and all six miR172 targets simultaneously.

In conclusion, SQN contributes to both stem cell homeostasis and floral stem cell termination. Although other targets of miR172, as well as other miRNAs, might also be involved, the influence of SQN on stem cell dynamics appears to be largely mediated by the miR172/AP2 tandem, which in turn modulates CLV signalling, and AG expression. Modulation of CLV signalling seems sufficient to explain the effect of SQN on stem cell homeostasis, but not on floral determinacy. SQN promotes stem cell termination in the flower by activating the expression of AG in the centre of the flower (Prunet et al.,
This activation of AG probably depends on both the CLV pathway, which has a positive effect on AG transcription in this domain (Clark et al., 1993), and the down-regulation of AP2, which is a direct repressor of AG (Drews et al., 1991; Jofuku et al., 1994; Yant et al., 2010). Overall, the present data shed new light on several important factors that contribute to the robustness of stem cell dynamics in Arabidopsis.

Supplementary data

Supplementary data are available at JXB online.

Figure S1. SQN mRNA level in wild-type and sqn mutant plants.

Figure S2. sqn-4 and clv-3-2 siliques with extra carpels developing above the base of the gynoecium.

Figure S3. sqn-4 inflorescence meristem branching after fasciation.

Figure S4. sqn-4 mutants exhibit an enlarged central zone.

Figure S5. Peduncles of senesced SQN 35S::CLV3 and sqn-4 35S::CLV3 flowers.

Table S1. sqn and miR172 alleles used in this study.

Table S2. Primer list.

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