Interaction between Phosphate-Starvation, Sugar, and Cytokinin Signaling in Arabidopsis and the Roles of Cytokinin Receptors CRE1/AHK4 and AHK3[1]

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Cytokinins control key processes during plant growth and development, and cytokinin receptors CYTOKININ RESPONSE 1/WOODEN LEG/ARABIDOPSIS HISTIDINE KINASE 4 (CRE1/WOL/AHK4), AHK2, and AHK3 have been shown to play a crucial role in this control. The involvement of cytokinins in signaling the status of several nutrients, such as sugar, nitrogen, sulfur, and phosphate (Pi), has also been highlighted, although the full physiological relevance of this role remains unclear. To gain further insights into this aspect of cytokinin action, we characterized a mutant with reduced sensitivity to cytokinin repression of a Pi starvation-responsive reporter gene and show it corresponds to AHK3. As expected, *ahk3* displayed reduced responsiveness to cytokinin in callus proliferation and plant growth assays. In addition, *ahk3* showed reduced cytokinin repression of several Pi starvation-responsive genes and increased sucrose sensitivity. These effects of the *ahk3* mutation were especially evident in combination with the *cre1* mutation, indicating partial functional redundancy between these receptors. We examined the effect of these mutations on Pi-starvation responses and found that the double mutant is not significantly affected in long-distance systemic repression of these responses. Remarkably, we found that expression of many Pi-responsive genes is stimulated by sucrose in shoots and to a lesser extent in roots, and the sugar effect in shoots of Pi-starved plants was particularly enhanced in the *cre1 ahk3* double mutant. Altogether, these results indicate the existence of multidirectional cross regulation between cytokinin, sugar, and Pi-starvation signaling, thus underlining the role of cytokinin signaling in nutrient sensing and the relative importance of Pi-starvation signaling in the control of plant metabolism and development.

Cytokinins are structurally diverse plant hormones with important roles in growth and development. Since their discovery as plant cell division promoting substances, an ever expanding number of roles have been attributed to these hormones. These include their well-established roles in the control of root branching and growth, shoot initiation, leaf differentiation, chloroplast biogenesis, and senescence (for review, see Mok and Mok, 1994, 2001; Haberer and Kieber, 2001). Several reports have also implicated cytokinins in responses related to the status of nutrients such as sugar, nitrogen, phosphorous, and sulfur (for review, see Sakakibara, 2003; Franco-Zorrilla et al., 2004; Maruyama-Nakashita et al., 2004a).

Significant progress has been recently made toward the elucidation of the molecular details of cytokinin signaling, leading to a model for signal transduction involving a His-Asp phosphorelay cascade that is similar to bacterial two-component systems (for review, see Hutchinson and Kieber, 2002; Hwang et al., 2002; Heyl and Schmulling, 2003). Molecular and genetic analyses have shown that the *CYTOKININ RESPONSE 1/WOODEN LEG/ARABIDOPSIS HISTIDINE KINASE 4* (*CRE1/WOL/AHK4*) gene encodes a cytokinin receptor (Mähönen et al., 2000; Inoue et al., 2001; Suzuki et al., 2001; Franco-Zorrilla et al., 2002). CRE1 is a hybrid His kinase that, upon perception of the hormone, is activated through autophosphorylation at a His residue and triggers a cascade of phosphorelay reactions. In Arabidopsis (*Arabidopsis thaliana*), there are two additional genes, *AHK2* and *AHK3*, coding for His kinase proteins with close homology to CRE1 (Inoue et al., 2001; Ueguchi et al., 2001; Yamada et al., 2001). The recent isolation and analysis of single, double, and triple mutants of members of the *CRE1* family have proven the important overlapping, but distinct, role of these cytokinin receptors in root growth, leaf development, formation and maintenance of meristem activity, and reproductive growth (Higuchi et al., 2004; Nishimura et al., 2004). However, the full physiological roles of cytokinins and cytokinin receptors are not yet understood, particularly with regard to their participation in the modulation of responses to plant nutrient status.

The involvement of cytokinins in nutrient signaling responses was first suggested by studies in which cytokinin levels were found to decrease after phosphate (Pi) or nitrate starvation (Salama and Wareing,

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1979; Horgan and Wareing, 1980). In the case of Pi, we have shown that exogenous application of cytokinins represses the induction of many Pi starvation-responsive genes (Martín et al., 2000), and this effect is impaired in *cre1* mutants, implicating cytokinin two-component signaling in the negative regulation of Pi-starvation responses (Franco-Zorrilla et al., 2002). Similar results regarding cytokinins and CRE1 have been reported for high affinity sulfate transporter genes (Maruyama-Nakashita et al., 2004b). The observation that cytokinins affect only Pi-starvation responses that are dependent upon whole plant Pi status (controlled by systemic signals), and not local Pi-dependent responses (such as increased root hair number and length), led us to suggest that the cytokinin signaling pathway could be a candidate for the systemic repression signaling system (Martín et al., 2000).

Cytokinin signaling may also be involved in sugar sensing. Loss-of-function mutations in the *HEXOKINASE 1* (*HXK1*) gene display decreased sensitivity to sugar as well as increased cytokinin sensitivity. Reciprocally, constitutive activation of cytokinin signaling confers decreased sugar sensitivity (Moore et al., 2003). In summary, there is good evidence for the interaction between cytokinins and the sensing of several nutrients, although its significance remains unclear. Moreover, links among the sensing of different nutrients are emerging, with sugar sensing occupying a central position. Indeed, interactions between sugar and nitrogen sensing have been documented (Coruzzi and Zhou, 2001; Price et al., 2004) and some reports indicate a relationship between sugars, sulfate, and Pi (Sadka et al., 1994; Nielsen et al., 1998; Ciereszko et al., 2001; Ciereszko and Kleczkowski, 2002; Hammond et al., 2003; Lejay et al., 2003; Wu et al., 2003; Maruyama-Nakashita et al., 2004a). Pi starvation, for instance, was shown to decrease the expression of several sugar-induced genes in different plants, and sugars have been shown to induce the expression of a Pi transporter in Arabidopsis (Sadka et al., 1994; Nielsen et al., 1998; Ciereszko et al., 2001; Ciereszko and Kleczkowski, 2002; Lejay et al., 2003). Additionally, microarray expression analysis of Pi-starved plants showed that the expression of genes involved in carbohydrate metabolism is altered under this stress (Hammond et al., 2003; Wu et al., 2003). All these data prompted us to investigate further the possible interactions between sugar, Pi, and cytokinin signaling.

In this study, we report the isolation of a mutant displaying reduced sensitivity to cytokinin repression of a Pi starvation-responsive gene and show that it corresponds to a mutant allele of the cytokinin receptor *AHK3*. This gene displays partial redundancy with *CRE1* in several cytokinin responses, including the repression of Pi-starvation responses, as well as sugar sensitivity. Studies on the expression of Pi starvation-responsive genes in wild type, and *cre1* and *ahk3* mutants, suggest that a prominent role for CRE1 and AHK3 in systemic repression of Pi starvation is unlikely. Remarkably, these studies provide evidence for a functional link between cytokinin, sugar, and Pi-starvation signaling, involving the CRE1 and AHK3 receptors.

RESULTS

Isolation and Characterization of a Cytokinin Hyposensitive *ahk3* Mutant

With the aim of identifying genes responsible for the repression by cytokinins of the expression of Pi starvation-responsive genes, we previously screened for mutants defective in the cytokinin-mediated repression of the Pi starvation-responsive *IPS1*-β-glucuronidase (*GUS*) reporter gene. In that study, we recovered 10 mutant lines, with seven displaying high cytokinin insensitivity and showing mutations at *CRE1* (Franco-Zorrilla et al., 2002). One of the three lines not corresponding to *CRE1* was less sensitive to cytokinin repression than the wild type at lower cytokinin concentrations (Fig. 1). This mutant exhibited insensitivity to this hormone in additional cytokinin response assays (see below; Fig. 1).

The mutation segregated as a codominant trait in F_2 segregating populations obtained from backcrosses with the wild type (not shown). Mapping of the mutation revealed that it was within a 1.1-Mb region on chromosome 1 flanked by simple sequence length polymorphism (SSLP) markers ZFPG and nga392. The *AHK3* gene is within this interval (At1g27320; Ueguchi et al., 2001) and sequence analysis revealed a G to A transition in this gene in the mutant line. This mutation generates a Ser to Phe change at amino acid 713 of the predicted AHK3 protein. This is located at the C-terminal end of the His kinase catalytic domain and within the G2 motif, which is involved in ATP/ADP binding (Fig. 1A; Stock et al., 2000; Wolanin et al., 2002). Transformation of the mutant with a 7.8-kb genomic fragment containing the *AHK3* gene rescued the wild-type phenotype in 11 out of 13 independent transgenic lines homozygous for the construct (data not shown). These results confirmed that the mutant was an allele of *AHK3*, which we have designated *ahk3-4* (hereafter referred to as *ahk3*). For further analysis, the *ahk3* mutant was backcrossed four times. To evaluate the effects of the *ahk3* mutation, we performed several phenotypic tests related to cytokinin responses. Given the high similarity between AHK3 and CRE1 (81% amino acid similarity; being the highest similarity among cytokinin receptors), these analyses were also carried out initially (i.e. experiments in Fig. 1) for two strong *cre1* alleles, *cre1-3* and *cre1-7* (Franco-Zorrilla et al., 2002), and the corresponding double mutants *cre1-3 ahk3* and *cre1-7 ahk3*. Due to the similar phenotypes shown by the two *cre1* alleles in these experiments, either alone or in combination with *ahk3*, experiments presented in subsequent sections were carried out only on the *cre1-3* allele (hereafter referred to as *cre1*) and the corresponding double mutant (*cre1 ahk3*). Here, we show only the results

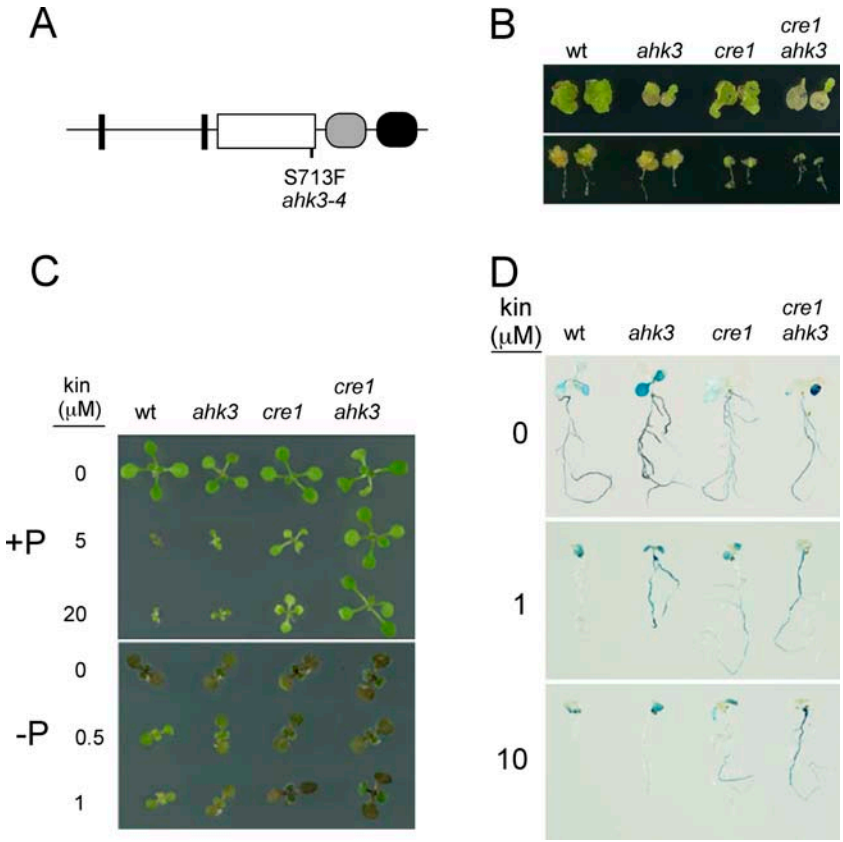

Figure 1. Isolation and characterization of the *ahk3* mutant. A, Scheme of the AHK3 predicted protein, where biologically relevant domains are represented as follows: transmembrane domains, black rectangles; His kinase, white box; pseudo-receiver domain, gray oval; and receiver domain, black oval. The point mutation in *ahk3-4* causing a Ser to Phe substitution is indicated. B, Calli growth assays. Cotyledon (top) and root (bottom) explants from the same lines as in A were assayed for calli formation in the presence of 100 ng mL^{-1} 2,4-dichlorophenoxyacetic acid and 100 (cotyledons) or 125 (roots) ng mL^{-1} kinetin. C, Plant growth and the effect of kinetin. Wild-type (wt), *ahk3*, *cre1*, and double *cre1 ahk3* mutants were cultivated for 9 d in Pi-sufficient (+P; top) and in Pi-deficient (−P; bottom) media supplemented with kinetin at different concentrations for 9 d. D, *IPS1-GUS* expression in wild-type (wt), *ahk3*, *cre1*, and double *cre1 ahk3* mutants. Plants were cultivated in Pi-lacking medium supplemented with kinetin for 9 d and histochemical analysis of GUS activity was performed.

from this *cre1* allele. We first examined callus formation and growth from cotyledon and root explants. Qualitative differences were observed between the *ahk3* and *cre1* mutants (Fig. 1B). *ahk3* cotyledons displayed higher insensitivity to cytokinins than cotyledons of *cre1*, whereas the opposite was found when root explants were used in these assays. For both types of explants, the response of the double mutant was lower than that of any of the single mutants. These results are very similar to those reported for putative loss of function T-DNA insertion alleles (Higuchi et al., 2004; Nishimura et al., 2004), suggesting that *ahk3-4* is also a strong (if not a loss of function) allele. Next, we evaluated plant growth under two Pi regimens (Pi sufficient and Pi starvation) in the presence of different amounts of cytokinin (kinetin). No difference was observed between the mutants and the wild type when grown in the absence of cytokinin (Fig. 1C). At all kinetin concentrations tested, *ahk3* plants showed only a slight insensitivity to the hormone, intermediate between that of *cre1* and wild-type plants. This is seen in the growth of rosettes, as well as in anthocyanin accumulation in Pi-starved plants, which in the wild type is dramatically reduced by cytokinins (Fig. 1C). However, the double mutant revealed more than additive effects of *cre1* and *ahk3* on the development of the plants grown in the presence of kinetin, which were much more conspicuous under high concentrations of the hormone (Fig. 1C). Similar results were obtained for the repression of the Pi-responsive *IPS1*:*GUS* reporter gene (Fig. 1D). Altogether, these results indicate overlapping but distinct functions of AHK3 and CRE1 and their involvement in the negative control of Pi-starvation responses by cytokinins.

Molecular Characterization of *ahk3*

To evaluate the effects of the *ahk3-4* mutation at the molecular level, we performed northern-blot analyses to monitor the expression of the primary cytokinin response genes A-type *ARABIDOPSIS RESPONSE REGULATOR*s (*ARR*s; Fig. 2A) and of Pi starvation-induced genes (Fig. 2B). Two sets of A-type *ARR* genes were used as markers: *ARR4* and *ARR6*, which are mainly induced in shoots (D'Agostino et al., 2000; To et al., 2004) and *ARR15* and *ARR16*, whose expression in roots is particularly reduced in *cre1-1* mutants (Kiba et al., 2002). Additionally, we examined the expression of the cyclin *CYCD3*, which has been shown to be induced by cytokinins after 24 h (Riou-Khamlichi et al., 1999). In the absence of hormone, basal expression of all the genes tested was quite similar in wild-type and mutant plants. However, after kinetin treatment, the induction of all these genes was compromised in mutant plants, albeit to different extents (Fig. 2A). In particular, *ARR16* showed a similar dramatic reduction

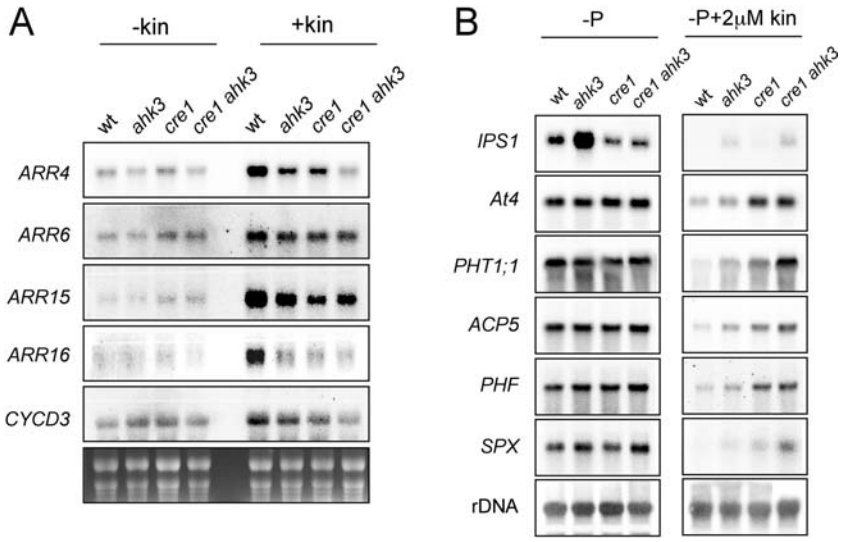

Figure 2. Molecular characterization of *ahk* mutants. A, Northern analysis of expression of type cytokinin-induced genes in wild type (wt), *ahk3*, *cre1*, and the corresponding double mutant (*cre1 ahk3*). RNA was extracted from plants grown for 6 d and treated with or without kinetin (15 μM) for an additional day. Total RNA was isolated and RNA blots containing 15 μg of RNA per sample were subsequently hybridized to probes corresponding to the cytokinin-induced genes indicated. B, Influence of kinetin on the expression of Pi starvation-responsive genes in roots. Plants were grown in absence of Pi supplemented or not with 2 μM kinetin, and root material was collected after 9 d. Total RNA was isolated from roots, and RNA blots containing 15 μg of RNA per sample were subsequently hybridized to probes corresponding to the Pi starvation-responsive genes indicated.

in expression in any of the single *cre1* and *ahk3* mutants and in the double mutant, suggesting that expression of this gene requires the cooperation between CRE1 and AHK3. Molecularly, such cooperation could be via the formation of a heterodimer, given the likely action of these receptors as dimers as suggested by interallelic complementation studies with *cre1* (*wol*) alleles (García-Ponce de León et al., 2004).

The effect of the *cre1* and *ahk3* mutations on *CYCD3* expression is noteworthy, since previously it was found that cytokinin responsiveness of *CYCD3* was dependent on regulatory phosphorylation (Riou-Khamlichi et al., 1999). Our finding suggests that regulatory phosphorylation acts downstream of canonical cytokinin signaling.

To test the effect of these mutations on the expression of Pi starvation-responsive genes, RNAs were obtained from roots of plants grown for 7 d in the absence of Pi and in the presence or absence of kinetin. We analyzed the expression of several Pi starvation-responsive genes, the related nonprotein coding genes *IPS1* and *At4* (Burleigh and Harrison, 1999; Martín et al., 2000), the high affinity Pi transporter gene *PHT1;1* (Muchhal et al., 1996), the purple acid phosphatase gene *ACP5* (del Pozo et al., 1999), and *PHF* and *SPX* (At3g52190 and At2g45130, respectively; F. Scaglia, R. Bustos, and J. Paz-Ares, unpublished data). In this experiment (Fig. 2B), all genes except *IPS1* responded similarly to Pi starvation in the absence of cytokinins in wild-type and mutant plants. However, in the presence of cytokinins, while Pi-starvation genes were dramatically down-regulated in the wild type, in *ahk3* and especially *cre1* mutants, and even more the *cre1 ahk3* double mutant, they displayed reduced down-regulation. These data support mostly additive roles of both receptors in the cytokinin repression of the Pi-starvation response. Further investigation is required to explain the higher expression of *IPS1* in *ahk3* and its reduced expression in *cre1*.

Systemic Down-Regulation of Pi-Starvation Responses in *ahk* Mutants

In roots, most nutrient deficiency responses, including Pi starvation, depend on the whole plant status of the nutrient in question rather than on the external concentration of the nutrient. As a result, if one part of the root system receives enough nutrient to satisfy the needs of shoot growth, the corresponding nutrient starvation response will be systemically down-regulated in the remaining part of the root system (see, for instance, Drew and Saker, 1984; Scheible et al., 1997; Liu et al., 1998; Burleigh and Harrison, 1999; Lappartient et al., 1999). We previously highlighted a parallel between this phenomenon and the repression of the Pi-starvation responses by cytokinins (Martín et al., 2000). To test the role of cytokinin signaling in systemic down-regulation of Pi-starvation responses, we conducted split root experiments with wild type and the double mutant *cre1 ahk3*, in which one part of the root system of Pi-starved plants was placed in Pi-rich media, whereas the other part was placed in media lacking this nutrient, as schematically represented in Figure 3A. We analyzed the expression of some Pi starvation-inducible genes in a time-course experiment by RNA-gel blot analysis (Fig. 3B). Repression of Pi starvation-induced gene expression in the roots of both genotypes was observed within the first day of the treatment, regardless of whether the split roots were grown in Pi-rich or in Pi-lacking media. In the double mutant, a slight decrease in repression could be observed, but relative to the wild type, it was not specific for the split roots growing in Pi-lacking media.

We also examined whether externally added cytokinins could trigger systemic repression using a similar split root assay with wild-type plants harboring the *IPS1-GUS* reporter gene (Fig. 4). When the roots of Pi-starved plants were split between Pi-containing and Pi-lacking media, the expression of the *IPS1-GUS* was

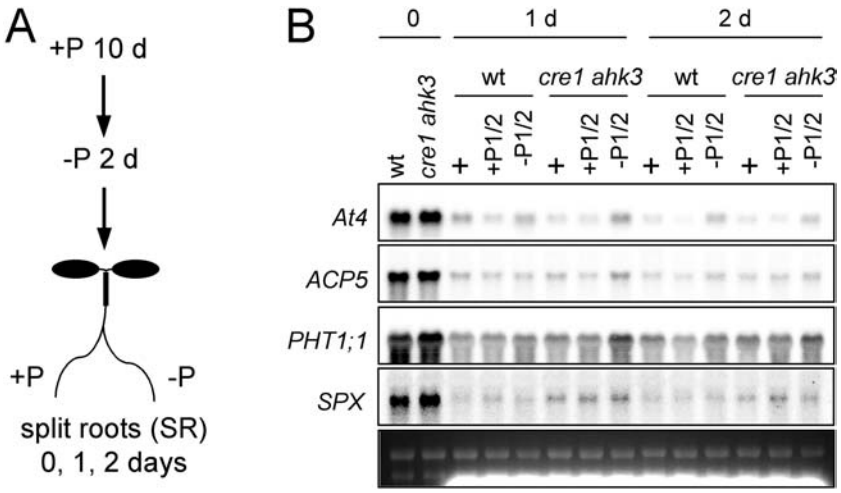

Figure 3. Split-root assays of systemic repression of Pi-starvation responses in *ahk* mutants. A, Schema of the split root experiment. First plants were grown for 10 d in complete medium and then transferred for an additional 2 d to a Pi-lacking medium. Subsequently, plants were transferred for 1 or 2 d to split plates with two independent compartments placing part of the root system in each of these compartments. B, Northern analysis of systemic repression of Pi-starvation response in the *cre1 ahk3* double mutant. Plants were grown for 1 or 2 d with the roots split in two compartments having Pi-sufficient (P1/2) media and Pi-lacking media (−P1/2), respectively. As control, both parts of the split root system were placed in Pi-rich media (+). In this experiment, only wild-type (wt) and double mutant *cre1 ahk3* RNA were prepared from each part of the split root harvested independently when they where placed in different media, and 10 μg of RNA sample were used in the experiment. The blot was hybridized with the probes corresponding to the Pi starvation-responsive genes indicated.

negligible in both parts of the root, indicating systemic down-regulation (Fig. 4A). In contrast, when the roots were split and transferred to media lacking Pi with or without cytokinin, then repression of *IPS1-GUS* occurred only in the part of the root system in contact with the hormone (Fig. 4B). This indicates that exogenously added kinetin does not translocate efficiently throughout the whole plant and that local perception of cytokinin is necessary to block the Pi-starvation response.

Sugar Sensitivity of *ahk* Mutants

Recently, an antagonistic interaction between cytokinin and HXK1-dependent sugar signaling has been described (Moore et al., 2003). We therefore investigated whether CRE1 and AHK3 could be directly involved in such sugar-cytokinin cross talk. We evaluated sugar sensitivity of single mutants, the double mutant *cre1 ahk3*, and a transgenic line highly overexpressing *AHK3* (driven from the cauliflower mosaic virus 35S promoter; see "Materials and Methods"). The overexpressing line used was chosen out of three showing the highest level of *AHK3* RNA, and concomitantly resulting in enhanced response (albeit moderate) to cytokinin in root growth assays and repression of *IPS1*:*GUS* expression (data not shown). Plants were germinated and grown in complete medium with varying concentrations of Suc for 10 d. Plants ectopically expressing *AHK3* were more resistant to high Suc concentrations than the wild type and, reciprocally, cytokinin-insensitive mutants displayed higher Suc sensitivity, in particular the *cre1 ahk3* double mutant (Fig. 5). Control experiments in which mannitol was substituted for the Suc were carried out in parallel, and no decrease in plant survival was observed, excluding differences attributable to osmotic effects (Fig. 5B). Thus, these results confirm the antagonistic interaction between cytokinin and Suc and involve *CRE1* and *AHK3* in this effect.

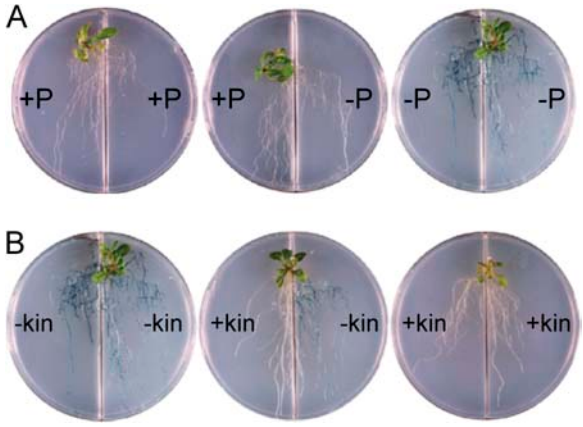

Figure 4. Effect of cytokinin on repression of Pi starvation-responsive *IPS1-GUS* in split root assays. Ten-day-old transgenic plants harboring the *IPS1-GUS* gene (Martín et al., 2000) were Pi starved for 2 d, then transferred to split plates with the compartments containing media with the compositions as indicated and, after 3 d, histochemical analysis of GUS activity was performed. A, The compartments had Pi-sufficient (+P) or Pi-deficient (−P) media. B, The compartments had media lacking Pi, but each was supplemented or not with 10 μM kinetin (kin).

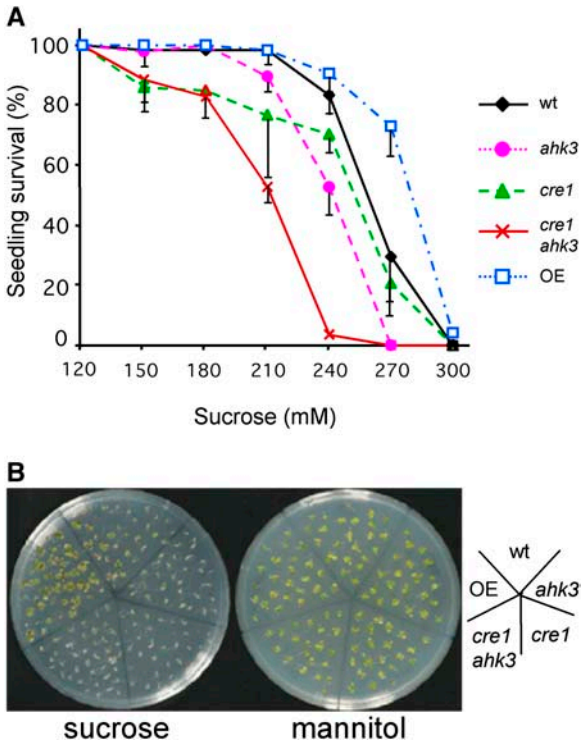

Figure 5. *ahk* mutants display Suc sensitivity. A, Seedlings were grown for 10 d in media with increasing concentrations of Suc in a range from 60 to 360 mM and scored after this time for seedling survival. Suc concentrations below 120 mM did not cause lethality in any of the genotypes tested and above 300 mM prevented seedlings survival. Genotypes used are the same as in previous figures and OE represents one *35S-AHK3* overexpressing line. Vertical bars represent SDS from two replica experiments in which 40 seedlings per genotype and experiment were scored. B, Ten-day-old plants from the same genotypes grown at 275 mM Suc or mannitol.

Sugar Control of Pi Starvation-Responsive Genes in *ahk* Mutants

There is some evidence supporting the interaction between sugar and Pi sensing (Sadka et al., 1994; Nielsen et al., 1998; Ciereszko et al., 2001; Ciereszko and Kleczkowski, 2002; Hammond et al., 2003; Lejay et al., 2003; Wu et al., 2003). These indications, together with the antagonistic interaction between sugar and cytokinins, prompted us to evaluate the effects of sugars and Pi starvation on gene expression in the cytokinin-insensitive mutants, as well as in the transgenic line overexpressing *AHK3*. For this purpose, RNA was extracted from plants grown for 7 d in the presence of low Suc and then transferred for 3 d to media lacking Pi and containing high or low Suc before harvesting shoots and roots separately (Fig. 6). Pi starvation-responsive genes whose expression was analyzed were the same as in previous experiments (Fig. 2), as well as *SQD1* (Essigmann et al., 1998) and *CaBP22* (At2g41090; F. Scaglia, R. Bustos, and J. Paz-Ares, unpublished data). Additionally, we analyzed the expression of two sugar-inducible genes, *APL3* and *β-AMY* (Mita et al., 1995; Sokolov et al., 1998).

Despite some differences in the behavior of the various genes analyzed, the overall picture is that high Suc enhances the expression not only of sugar-induced genes but also of Pi starvation-responsive genes, particularly in shoots, but also in roots (when expression was detected), and the effect of sugars in shoots of Pi-starved plants was particularly enhanced in the *cre1 ahk3* double mutant (Fig. 6). We note that shoots are sensitive to Pi starvation and in fact the expression of several of the genes examined in this experiment (*At4*, *IPS1*, *ACP5*, *PHT1;1*, and *SQD1*) has been demonstrated to be Pi starvation responsive in shoots (Muchhal et al., 1996; Essigmann et al., 1998; Burleigh and Harrison, 1999; del Pozo et al., 1999; Martín et al., 2000). The nonprotein coding genes, *At4* and *IPS1*, deviated from the behavior displayed by all other Pi starvation-responsive genes examined, although they showed differences in expression levels in mutants relative to wild-type plants, at least in some of the conditions/tissue tested and displayed responsiveness to sugars (at least in some mutants). The reasons for the different behavior of these presumably regulatory, nonprotein coding genes, needs to be further investigated.

Some differences were observed between the expression of sugar-responsive and Pi starvation-responsive genes in relation to the *cre1* and *ahk3* mutants. Thus, the expression of sugar-responsive genes was more highly enhanced in the double mutant than Pi starvation-responsive genes, and *ahk3* had a negligible contribution to the increase in expression of these genes, whereas in the case of Pi starvation-responsive genes, the effect of *ahk3*, although not so important as that of *cre1*, was evident (Fig. 6).

In the cases in which the effect of the *cre1* or *ahk3* mutations on overall gene expression was not evident (i.e. in shoots from low-Suc grown plants and in roots from high- or low-Suc grown plants), *AHK3* overexpression resulted in a small but consistent decrease of the expression of all sugar and Pi starvation-inducible genes. Altogether, these results provide a clear indication of an interaction between cytokinin, sugar, and Pi-starvation signaling and suggests that the role of cytokinin signaling in the control of sugar and Pi starvation-responsive genes may be quite broad and complex and not exclusive for shoots of plants grown under a high-Suc, low-Pi regimen.

DISCUSSION

Many studies have shown that plants have nutrient-specific signaling mechanisms to adapt their growth and development to changing nutritional conditions. One example of these is the controlling system of Pi starvation, in which the transcription factor PHR1 plays a key regulatory role (Rubio et al., 2001). This study reveals an additional level of complexity in the control of Pi-starvation responses, as indicated by the multidirectional interactions between Pi starvation,

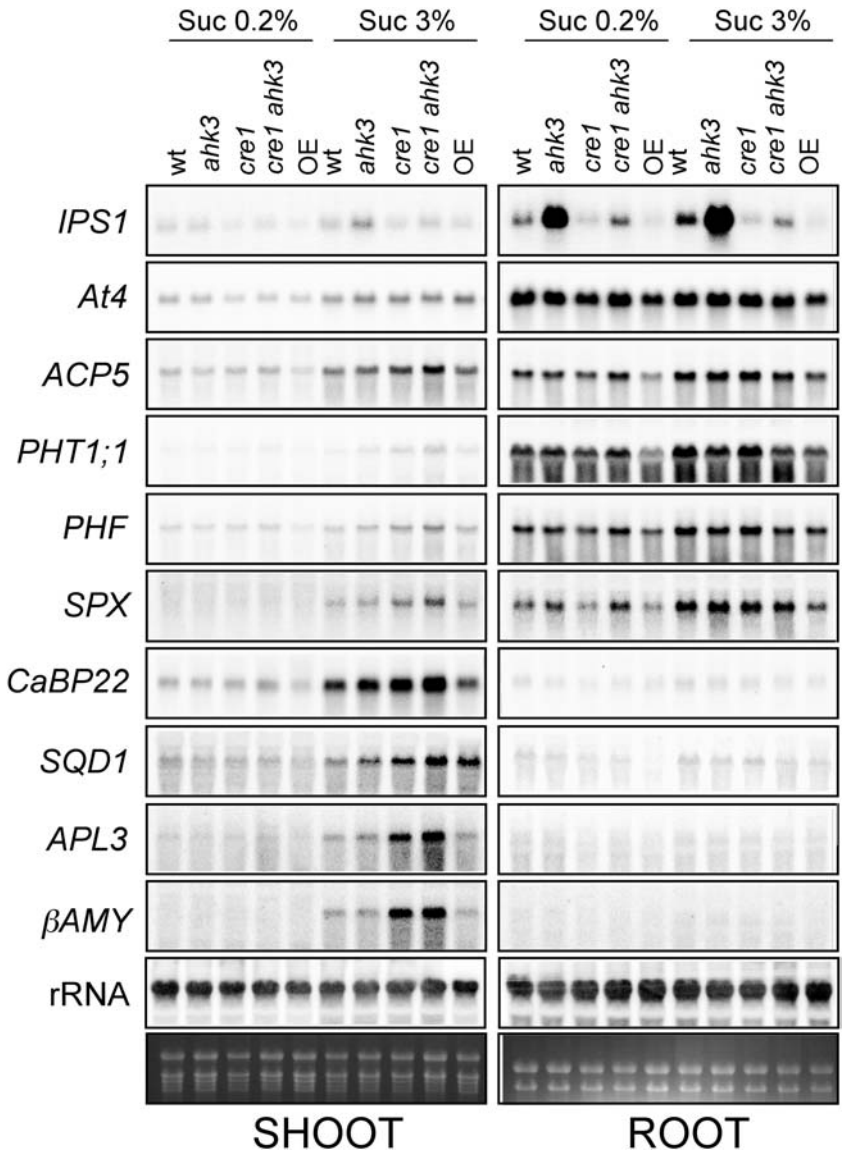

Figure 6. Effect of Suc on the expression of Pi starvation-responsive genes in *ahk* mutants. Plants of the genotypes indicated were grown for 7 d in Pi-rich media containing a low Suc concentration (0.2%) and transferred for additional 3 d to Pi-lacking media containing low (0.2%) or high (3%) Suc concentration. RNA was prepared from shoots and roots separately and 15 μg was loaded in each lane. Blots were hybridized to the probes corresponding to Pi starvation-responsive genes (*IPS1*, *At4*, *ACP5*, *PHT1;1*, *PHF*, *SPX*, *CaBP22*, and *SQD1*) and sugar-responsive genes (*APL3* and *β-AMY*).

sugar, and cytokinin signaling, involving the cytokinin receptors CRE1 and AHK3. This finding further underlines the relevance of cytokinins in relation to nutrient responses and the prominent role of Pi-starvation signaling in the control of plant metabolism and development.

Cytokinin Interactions with Sugar and Pi-Starvation Signaling

Our studies have shown that plants with impaired cytokinin receptors CRE1 and AHK3 display increased sugar sensitivity in seedling survival tests and enhanced expression of both Pi starvation- and sugar-responsive genes in shoots of high sugar grown plants (see ''Results'' and Figs. 5 and 6). In the case of sugar signaling, it has recently been reported that there is a bidirectional antagonistic interaction between sugars and cytokinins. In this study, however, the effect of cytokinins on sugar sensing was based on studies with transgenic plants overexpressing genes constitutively activating cytokinin signaling (Moore et al., 2003). Our results showing that the *cre1-3* and *ahk3-3* mutants, particularly in combination, display increased Suc sensitivity both in seedling survival and gene expression assays provide a clear demonstration on the involvement of these two genes in the sugar cytokinin interaction.

It is noteworthy that our study revealed an effect of the *cre1* mutation on the *β-AMY* expression, whose sugar responsiveness has been attributed to the HXK1-independent sugar-sensing pathway (Xiao et al., 2000). This extends the effect of cytokinins on sugar-responsive genes beyond the sugar sensing dependent of HXK1

(Smeekens, 2000; Rolland et al., 2002; Moore et al., 2003), at least during Pi starvation. A similar conclusion on a bidirectional interaction can be drawn for Pi starvation and cytokinins, since it was shown that Pi starvation reduces cytokinin signaling by decreasing both cytokinin content and *CRE1* expression (Salama and Wareing, 1979; Horgan and Wareing, 1980; Franco-Zorrilla et al., 2002).

It is intriguing that the effect of *cre1* and *ahk3* mutations on the expression of sugar and Pi starvation-responsive genes is only evident under conditions of high sugar and in the shoot, but not in the root (Fig. 5). Such a restricted role of cytokinins in Pi-starvation signaling is in conflict with reports associating the increase of the root-to-shoot growth ratio of plants during Pi starvation with the observed reduction of cytokinin signaling under these conditions (Kuiper, 1988; Kuiper et al., 1988). In addition, it is plausible that senescence, a Pi-mobilizing condition that can be stimulated by Pi starvation, involves reduced cytokinin signaling. A simple explanation of these apparent differences is that there is additional cytokinin receptor function in the *cre1 ahk3* double mutant probably mediated by the third cytokinin receptor, AHK2, whose partial functional redundancy with CRE1 and AHK3 is differentially manifested in different parts of the plant and under different sugar regimens. This possibility would be in line with the recently reported existence of both redundant and specific functions for *CRE1*, *AHK2*, and *AHK3* in relation to plant growth and development (Higuchi et al., 2004; Nishimura et al., 2004). Actually, the fact that Pi starvation-responsive genes are repressed by cytokinins in roots (Martín et al., 2000; see also Figs. 1, 2, and 3) and the existence of some cytokinin sensitivity in the double *cre1 ahk3* mutant, at least concerning cytokinin repression of Pi starvation-responsive genes (Figs. 1 and 2), suggests that *AHK2* also plays a role in the control of nutrient related responses. Moreover, hyperexpression of *AHK3* does result in consistent, albeit moderate, reduction of nutrient-responsive gene expression both in roots, independent of the sugar concentration in the growth media, and to some extent also in shoots from low sugar grown plants (see Fig. 6). The analysis of the triple mutant *cre1 ahk2 ahk3* could confirm this hypothesis on the role of AHK2 regarding nutrient related responses. However, the severe growth and developmental defects of the triple mutant (Higuchi et al., 2004; Nishimura et al., 2004) complicates the analysis of Pi-starvation and sugar responses. The isolation of weak mutants of these *AHK* genes might help to overcome these limitations.

We have also investigated the potential role of cytokinins in long-distance repression signaling of Pi-starvation responses. However, the analysis of systemic repression remaining in the *cre1 ahk3* mutant did not support any significant role for these receptors per se in long-distance signaling of whole Pi status (Fig. 3). Additionally, exogenous cytokinins themselves are unable to systemically repress Pi-starvation responses (Fig. 4). Altogether, these results make a prominent role of cytokinins in systemic repression unlikely.

Interaction between Pi Starvation and Sugar Signaling

Phosphorus is an essential macronutrient, and plants have evolved an adaptive system to cope with growth under P limiting conditions, involving both developmental and metabolic adaptations (for review, see Raghothama, 1999; Abel et al., 2002; Rausch and Bucher, 2002; Franco-Zorrilla et al., 2004). A primary determinant of metabolic adaptation is the close linkage between Pi, the form in which phosphorus is assimilated, and sugar metabolism. Pi plays a key role in the coupling of light and dark reactions in photosynthesis and in the export of trioses from the chloroplasts. Pi is also a substrate or a product in many reactions of sugar metabolism and an effector of key enzymes of starch and Suc synthesis. Paralleling this close metabolic link, it was previously shown that Pi starvation induced the expression of several Suc-responsive genes (Nielsen et al., 1998; Sadka et al., 1994; Ciereszko et al., 2001; Ciereszko and Kleczkowski, 2002). Our results showing that expression of most if not all the Pi starvation-induced genes is enhanced by Suc (Fig. 6) indicate that the Pi-starvation/sugar regulatory interaction is bidirectional and not exclusive of the high affinity Pi transporter previously reported (Lejay et al., 2003).

One possible explanation for the highest expression of sugar and Pi starvation-responsive genes when Pi-starvation and high sugar conditions are combined could be that these genes are actually Pi starvation responsive and that high sugar further reduces cellular Pi levels by increasing the levels of sugar Pi (Sadka et al., 1994). However, this metabolic interpretation involving a Pi-starvation signal is insufficient alone. For instance, several mutants have been isolated displaying increased sugar sensitivity as assayed not only by the expression of sugar-responsive genes whose expression is enhanced by Pi starvation, such as *β-AMY* and *APL3*, but also in other sugar sensitivity assays, and these mutants do not display increased hexose content (Baier et al., 2004). Moreover, the cytokinin to sugar and cytokinin to Pi-starvation regulatory interactions mentioned above provide evidence for a formal link between sugar and Pi-starvation signaling, at least through cytokinin signaling.

Interplay between Cytokinin, Sugar, and Pi Signaling

In summary, our results demonstrate the bidirectional antagonistic interactions between cytokinin and both sugar and Pi-starvation signaling involving *CRE1* and *AHK3* and probably *AHK2*, as well as a positive bidirectional interaction between sugar and Pi-starvation signaling. These intricate interconnections between cytokinin, sugar, and Pi-starvation signaling place Pi-starvation signaling high in the regulatory hierarchy controlling plant metabolism and develop-

ment in accord with the physiological importance of Pi. Such regulatory cross talk allows not only Pi-starvation responses and Pi acquisition to be fine-tuned according to the status of the key signaling metabolites, sugars (whose rate synthesis is primarily determined by factors affecting photosynthesis such as light, CO_2, nitrate, cytokinins), but also metabolism and development to be adjusted to Pi status. For instance, low Pi will enhance sugar responses, such as those leading to starch production and releasing Pi from sugar Pi, and reduce cytokinin signaling, thereby increasing the root-to-shoot growth ratio and concomitantly the soil Pi scavenging potential, as well accelerating senescence, a Pi-mobilizing process. The engineering of plants for better Pi use efficiency will be dependent on appreciating this regulatory cross talk and the molecular mechanisms that underpin it.

MATERIALS AND METHODS

Plant Material

Arabidopsis (*Arabidopsis thaliana*) L. Heynh ecotypes used in this study were Columbia and Landsberg *erecta*. Growth conditions and media were as previously described (Franco-Zorrilla et al., 2002) except where indicated for Suc or mannitol concentrations.

Genetic Analysis and Positional Cloning of *AHK3*

ahk3-4 mutant plants were backcrossed four times to wild-type plants (Columbia). To map the mutation, we obtained an F_2 segregating population derived from a cross between the mutant and the Landsberg *erecta* ecotype. DNA was prepared from 48 plants showing the mutant phenotype and used to analyze linkage of the *ahk3* mutation to previously described SSLP (Bell and Ecker, 1994) and cleaved amplified polymorphic sequence (CAPS; Konieczny and Ausubel, 1993). A*HK3* was mapped to chromosome 1, between SSLP markers ZFPG and nga392, which defined a 1.1-Mb region.

Binary Constructs and Plant Transformation

A 7.8-kb genomic DNA fragment containing the *AHK3* gene and 2,628 upstream the ATG start codon and 915 bp and downstream the stop codon was obtained by PCR using bacterial artificial chromosome F17A16 DNA as template and Expand High Fidelity Polymerase (Roche Applied Science, Mannheim, Germany). The oligonucleotides employed were GTTTCCgtcgACTACATTCACGAAGTGCAAGG and AAGGGgTCGacTACTGCAACTCACCGTGAACG, where some nucleotides were substituted (small letters) to generate *Sal*I recognition sites (underlined). The PCR product was digested with *Sal*I and cloned in the *Sal*I site of the pCAMBIA1300 vector, generating *gAHK3*. The vector *gAHK3* was introduced into the C58 strain of *Agrobacterium tumefaciens* and Arabidopsis plants were transformed as described (Bechtold et al., 1993). Transgenic plants were selected on Murashige and Skoog medium supplemented with hygromicin (40 μg/mL) and carbenicillin (50 μg/mL). To obtain a cDNA fragment corresponding to *AHK3*, a two-step procedure was followed. First, two overlapping cDNA fragments corresponding to the 5′ and 3′ halves of *AHK3* were obtained through reverse transcription-PCR, using *AHK3*-specific oligonucleotides CGGAATTCCGAGAATATGGGCTGG and CTATAAACAAGTTCACATAAGG in the same reaction from 10 mg of RNA from seedlings as template and using Superscript Reverse Transcriptase (Ambion, Austin, TX). First strand cDNAs were subjected to PCR with oligonucleotide pairs GGGGGTTGATCGTGTATTCAAGTGGTGGATGCCAACCTTGGGAGTACTCGAGAATCC (5′-AHK3) and ATATCCAGGACGCATGGAGGCACAGG-ACTACTTCAAGATGCATAGG (3′-AHK3) using Expand High Fidelity Polymerase. PCR products were cloned into pGEMT vector (Promega, Madison, WI). In a second step, the 3′-AHK3 fragment was excised with *Xho*I-*Not*I and cloned into the *Xho*I-*Not*I sites of 5′-AHK3, generating an in-frame fusion for the complete *AHK3* cDNA. Finally, *AHK3* cDNA was excised with *Sma*I and *Not*I, blunt ended with Klenow (Roche), and cloned into the *Sma*I site of the pBIB vector downstream the 35S-cauliflower mosaic virus promoter (Becker, 1990) and introduced in *A. tumefaciens* (Bechtold et al., 1993).

Cytokinin Response Assays

In the calli induction experiments, plants were grown in complete medium for 7 d and cotyledons or 1-cm root pieces were excised and placed onto calli induction media as indicated in the text. For RNA-blot analysis of kinetin sensitivity, plants were grown in Murashige and Skoog for 6 d and transferred to Whatman paper soaked in liquid Murashige and Skoog medium supplemented or not with 15 μM kinetin for an extra day and RNA prepared from whole seedlings.

Northern Analysis and Probes

RNA extraction was carried out with the RNAwiz reagent (Ambion) following manufacturer's instructions. RNA electrophoresis, transfer to nylon membrane, and hybridization were performed following standard procedures (Sambrook et al., 1989). Genes analyzed included *AHK3* and *CRE1* (Franco-Zorrilla et al., 2002); the cytokinin-induced *ARR4*, *ARR6*, *ARR15*, *ARR16*, and *CYCD3* (Riou-Khamlichi et al., 1999; D'Agostino et al., 2000; Kiba et al., 2002; To et al., 2004); the Pi starvation-responsive *At4*, *IPS1*, *ACP5*, *AtPT1*, *SQD1* (Muchhal et al., 1996; Essigmann et al., 1998; Burleigh and Harrison, 1999; del Pozo et al., 1999; Martín et al., 2000), and *PHF*, *SPX*, and *CaBP22* (At3g52190, At2g45130 and At2g41090, respectively; F. Scaglia, R. Bustos, and J. Paz-Ares, unpublished data); and the sugar-induced genes *APL3* and *β-AMY* (Mita et al., 1995; Sokolov et al., 1998). Probes corresponding to *ARR6* and *ARR15*, *IPS1*, *At4*, *PHT1;1*, *ACP5*, *CRE1*, *APL3*, and *β-AMY* were obtained as previously described (Martín et al., 2000; Franco-Zorrilla et al., 2002; Baier et al., 2004; García-Ponce de León et al., 2004). Probes for *AHK3* and to *PHF* were amplified from cDNA with the following oligonucleotide pairs: ATATCCAGGACGCATGGAGGCACAGG-CTTAAGCAATGAGATTGCC (*AHK3*) and ATGGAGATTGAAGAAGCGAG-TTACATAATCTTTCTATAGG (*PHF*). Probes for *ARR4*, *ARR16*, *CYCD3*, *SPX*, *CaBP22*, and *SQD* were PCR amplified from genomic DNA with oligonucleotide pairs: GCTCGTCTATGGCCAGAGAC-CCAGAATAGTTCCACTAATC (*ARR4*); ATGGCTCTCAGAGATTTATC-TTGAGCTCAATCATTTAACC (*ARR16*); TTTAGTCCCCCACAATGGCG-TCGAGCTTTCGATTATGGAG (*CYCD3*); GATGAAGTTTGGAAAGAGG-GACAACAACATCATGGAATAGG (*SPX*); TCAAGGCCGAGAGTTCGTAG- TCTAACATACCAGCCAGAGG (*CaBP22*); ATGGCGCATCTACTTTCAGC- ACACAGAACCGGTTAAGTGC (*SQD1*).

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