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STIMPY mediates cytokinin signaling during shoot meristem establishment in *Arabidopsis* seedlings

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SUMMARY

The establishment of the primary meristems through proliferation after germination is essential for plant post-embryonic development. Cytokinins have long been considered a key regulator of plant cell division. Here we show that cytokinins are essential for early seedling development of *Arabidopsis*. Loss of cytokinin perception leads to a complete failure of meristem establishment and growth arrest after germination. We also present evidence that cytokinin signaling is involved in activation of the homeobox gene *STIMPY* (*STIP* or *WOX9*) expression in meristematic tissues, which is essential for maintaining the meristematic fate. Cytokinin-independent *STIP* expression is able to partially compensate for the shoot apical meristem growth defects in mutants that cannot sense cytokinin. These findings identify a new branch of the cytokinin signaling network, linking cytokinin to the process of meristem and seedling establishment.

KEY WORDS: *Arabidopsis*, *STIMPY*, Cytokinin signaling, Meristem

INTRODUCTION

Flowering plants initiate most of their organs post-embryonically from groups of pluripotent cells residing in the meristems. The primary meristems originate from embryogenesis, when the shoot apical meristem (SAM) and the root meristem are patterned and set aside (Steeves and Sussex, 1989). The patterning of the meristems has been extensively studied in *Arabidopsis* in the last two decades (reviewed in Baurle and Laux, 2003; Bowman and Floyd, 2008; Carles and Fletcher, 2003; Petricka and Benfey, 2008; Veit, 2004). Apart from the meristem-specific mechanisms, it has been demonstrated that general cell cycle regulators are involved in the maintenance of both the shoot and the root meristems (Blilou et al., 2002; Cockcroft et al., 2000; Riou-Khamlichi et al., 1999; Wildwater et al., 2005; Willemsen et al., 1998).

Although patterned at the embryonic stage, the meristems are only fully established during seedling development. The SAM enters active proliferation after germination, reaching its full size by the end of vegetative development (Medford et al., 1992). In the root, the fast-dividing meristematic zone also goes through a similar expansion following germination. Compared with the patterning aspect, mechanisms underlying the growth and establishment of the primary meristems in the emerging seedlings are less well understood.

One possible regulator of this process is the plant hormone cytokinin, which refers to a family of adenine derivatives with zeatin being the most abundant form. Cytokinins were first discovered for their ability to promote cell division in tissue culture (Miller et al., 1955) and have been linked to a wide range of developmental events (reviewed in Kyozuka, 2007; Mok and Mok, 2001). In intact plants, whereas the endogenous levels of cytokinins promote cell division, exogenously applied cytokinins in higher concentrations result in growth inhibition. This concentration-dependent effect is also reflected in how the primary meristems respond to changes in cytokinin signaling levels. Earlier studies have shown that in plants with partial loss of cytokinin availability or sensing, the two primary meristems often respond in opposite manners, leading to the hypothesis that cytokinins promote shoot growth while negatively regulating the size of the root meristem (Riefler et al., 2006; Werner et al., 2003). However, seedlings lacking all three cytokinin receptors show reduction in both the shoot and the root meristems, suggesting that low levels of cytokinin signaling positively regulate meristematic cell proliferation in both the above- and under-ground tissues (Higuchi et al., 2004; Nishimura et al., 2004), and the root meristem is inhibited by a lower threshold of cytokinins than the SAM (To and Kieber, 2008). Recently, it has been demonstrated that cytokinins regulate the size of the root meristems by controlling differentiation (Dello Ioio et al., 2007).

At the molecular level, cytokinin signal transduction is initiated by cytokinin binding to its two-component element receptors, the ARABIDOPSIS HISTIDINE KINASES (AHKs), triggering a cascade of phosphorelay events involving the ARABIDOPSIS RESPONSE REGULATORS (ARRs). The ARR proteins are divided into two groups based on their amino acid sequences and functional domains (D'Agostino and Kieber, 1999). The type-B ARR proteins, which are activated by changes in their phosphorylation state, positively regulate cytokinin responses by activating the transcription of their downstream targets, including the type-A *ARR* genes. The type-A ARR proteins then downregulate the cytokinin responses through a negative-feedback loop (reviewed in To and Kieber, 2008). More recently, a group of AP2 transcription factors, the CYTOKININ RESPONSE FACTORS (CRFs), have been found to be regulated by cytokinin signaling and involved in embryonic and cotyledon development (Rashotte et al., 2006).

The cytokinin signaling network interacts with known regulators of meristem development. In the shoot meristem, *WUSCHEL* (*WUS*) (Laux et al., 1996; Lenhard and Laux, 1999) represses the transcription of several type-A *ARRs*, thereby enhancing cytokinin activities (Leibfried et al., 2005). In the root, cytokinin signaling interacts with the auxin pathway in the establishment of the

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embryonic root meristem (Muller and Sheen, 2008). However, mutations in known cytokinin signaling targets do not phenocopy the defects seen in cytokinin perception mutants in their above-ground development (Argyros et al., 2008; Hutchison et al., 2006; Mason et al., 2005), implying additional mechanisms linking cytokinin signaling to shoot meristem development.

Previously, we reported that STIMPY [STIP; also known as WUSCHEL HOMEOBOX 9 (WOX9)] (Haecker et al., 2004; Wu et al., 2005), a homeodomain transcription factor that is related to *WUS*, plays a unique role in maintaining pluripotency and proliferation in meristematic tissue in *Arabidopsis*. At the seedling stage, the loss of *STIP* function does not cause any disruption in meristem patterning. Instead, the meristematic tissues in *stip* mutant seedlings exit the cell cycle and enter premature differentiation, resulting in the arrest of seedling development. This phenotype can be fully rescued by the addition of sucrose in the growth media. Genetically, *STIP* exerts a positive influence on *WUS* in regulating the stem cell population in the SAM (Wu et al., 2005). Here, we present evidence that cytokinin signaling is essential for early seedling development and that *STIP* acts downstream of cytokinin-sensing in the establishment of the SAM during this period.

MATERIALS AND METHODS

Plant materials

Plants were grown in long days (16 hours light/8 hours darkness) under about 120 μE m^{-2} sec^{-1} light at 22°C. To observe seedling phenotypes, seeds were germinated on 1/2 Murashige Minimal Organics Medium (MS; Phytotechnology Lab) with 0.6% agar after two days of stratification at 4°C. Sucrose (1.5%) was added to the media when the sugar effect was assayed.

stip-1 and *stip-2* were described previously (Wu et al., 2005). The ARR5::GUS reporter was obtained from the *Arabidopsis* stock center, and crossed to *stip-2*/+. The phenotypes were analyzed in F2.

Cytokinin sensitivity was assayed by transferring two-day-old *Col-0* and *stip-1* seedlings germinated on MS agar to MS agar with 1.5% sucrose and various concentrations of trans-zeatin (Sigma Z0876). The phenotypes were recorded after seven days.

In zeatin induction experiments, two-day-old seedlings were treated with 250 nM trans-zeatin on MS agar (3 ml per 100 mm plate). The control plates received 3 ml of water with 0.3 μl of acetic acid (same amount as in 3 ml of 500 nM trans-zeatin) each. Samples were incubated for 30 minutes before harvesting. For the zeatin-induction time course, two-day-old seedlings were treated with 250 nM trans-zeatin on MS agar, and the 0-minutes plates received no trans-zeatin.

Plasmid construction

pAS1 was generated by inserting the 5.3 kb *Eco*RI/*Bam*HI plasmid rescued genomic fragment from *stip-D* (Wu et al., 2005) into the transformation vector *pJHA212G* (Yoo et al., 2005). The segregating population of *ahk2-2 ahk3-3/+ cre1-12* was transformed using floral dipping (Weigel and Glazebrook, 2002). Transgenic plants were selected on MS agar with gentamicin (50 μg/ml). Seven independent single-insertion lines in *ahk2-2 ahk3-3/+ cre1-12* were selected from a total of 24 in the T2 generation and grown to T3, when plants homozygous for the *pAS1* insertion were analyzed for their ability to rescue the *ahk2-2 ahk3-3 cre1-12* mutants. All seven lines showed the same partial rescue effect.

The ethanol-inducible XW119 was generated in two steps. First, full-length *STIP* cDNA was introduced into the *Eco*RI site of *pBJ36-AlcA*. Then the *Not*I fragment of *AlcA::STIP:OCSt* was inserted into the transformation vector *pBart-35S::AlcR* (Roslan et al., 2001). Transgenic plants were selected on soil using Finale (Bayer).

Histological and morphological analysis

Wholemount in situ analysis was performed as previously described (Friml et al., 2003), except 10 μg/ml of proteinase K (Sigma-Aldrich # P2308) was used for digestion. For in situ hybridization on tissue sections, two-day-old seedlings were fixed in 4% paraformaldehyde and 0.1% Tween-20 in PBS for 5 hours, then dehydrated and infiltrated with paraffin using an H2850 Microwave Processor (Energy Beam Sciences) as previously described (Schichnes et al., 1999) with the following modifications: sections were digested with 20 μg/ml of proteinase K (Sigma-Aldrich # P2308) for 15 minutes, washed with 0.2× SSC for 1 hour at 55°C and the RNase treatment step was omitted. The *STIP* antisense RNA probe was described in Wu et al. (Wu et al., 2005). The antisense *ARR5* RNA probe was generated using the last exon of *ARR5* as the template.

GUS activity staining was carried out as previously described (Sessions et al., 1999), using 2 mM potassium ferro and ferri cyanide. The GUS-stained seedlings were mounted in 30% glycerol for analysis. The SAM sectioning and staining was done according to Roeder et al. (Roeder et al., 2003).

Tissue sections, in situ hybridization and GUS-stained samples were photographed on a Zeiss Axio Imager equipped with an AxioCam HRc camera. Seedling samples were photographed using an Olympus SZ61 stereo microscope with a Micrometrics camera. All comparative samples were taken under identical magnifications.

Microarray gene expression profiling

For the ethanol induction of *STIP*, four-day-old XW119 seedlings were treated with 2% ethanol on MS agar (3 ml/100 mm plate) and incubated at 22°C in light for 0, 1, 2 and 4 hours before harvesting. The 0-hour sample received no ethanol. Total RNA was extracted using the Spectrum Total Plant RNA (Sigma, STRN50-1KT). Biotin-labeled cDNA probes were generated using the One-Cycle Target Labeling and Control Reagents (Affymetrix) and hybridized to the ATH1 expression arrays. Each timepoint was done in triplicate.

Raw intensity data was pre-processed and normalized using gcRMA (Wu et al., 2004) and genes with unreliably low expression levels (<60) were filtered out. Differentially expressed genes were then identified using RankProd (Hong et al., 2006) at a *P*-value of 0.01. The raw data for the XW119 ethanol induction experiment is available at ArrayExpress, accession number E-MEXP-2229.

Quantitative RT-PCR

Total RNA was extracted using Spectrum Total Plant RNA (Sigma). The first-strand cDNA was obtained using SuperScript III First Strand (Invitrogen, 18080-051). Real-time PCR was carried out with the SYBR Green (Molecular Probes) method on Opticon-2 MJ machines. The relative changes in gene expression levels were determined using the $2^{-\Delta\Delta CT}$ method. Each treatment was done in at least two biological replicates, each with technical replicates. The primers used in this study are: *ARR5*, 5′-tcgata*tagatttagagggtattgtttg-3′ and 5′-ttaacggtaactttatcacaaatcctaa-3′; *ARR6*, 5′-tttgag*gatttgaaggttaatttaatt-3′ and 5′-caccggtacttctctaaaagctga-3′; *ARR7*, 5′-cgtatacaaga*atgtctcaaagaagga-3′ and 5′-taagattttgcattcctcagctt-3′; *ARR9*, 5′-actgatagtaacgacccaaatgc-3′ and 5′-atatctctaaaagctgatg-attc*cttg-3′; *CRF2*, 5′-tttccacctagagaagaagaagagtatc-3′ and 5′-gtttat-aacatagagattagggaaagcaaa-3′; *CRF5*, 5′-tgagtggtttgatgacaacg-3′ and 5′-cagataaataa*aaactccgttgcg-3′; *At4g27410*, 5′-tctggtcttttacgccggaaaa-3′ and 5′-cccaatcatccaa*cttggagct-3′; *At1g68670*, 5′-aggagcgtgta*atttgcaacc-3′ and 5′-agctttaatcaaatcacttttaccacc-3′; *ARR1*, 5′-cggaagaac*gatggacttacctg-3′ and 5′-gttactccctttagaaccacactcttt-3′; *ARR10*, 5′-gactatggagcaagaa-attgaagtct-3′ and 5′-ttcgttgttgtaa*cgtgatattgg-3′; *ARR12*, 5′-ggata-tgaga*aggctatgcctaaa-3′ and 5′-ctgattagccacaccactgatc-3′; *STIP*, 5′-tcaagttcaagcggacgca-3′ and 5′-caacctcttccccaaatgca-3′; *Act8*, 5′-ttccagcag*atgtggatctcta-3′ and 5′-agaaagaaatgtgatcccgtca-3′. The *Act8* primers have been previously described (Puthiyaveetil and Allen, 2008) and the asterisk (*) indicates exon-intron boundaries.

RESULTS

Cytokinin perception mutants fail to initiate seedling development

Previous studies found that *Arabidopsis* seedlings lacking all three cytokinin receptors, *AHK2*, *AHK3* and *CRE1* (*AHK4*), have reduced shoot and root meristems and impaired growth (Higuchi et al., 2004; Nishimura et al., 2004). However, in both cases, the seedlings were initially grown on sucrose-containing media, which can potentially

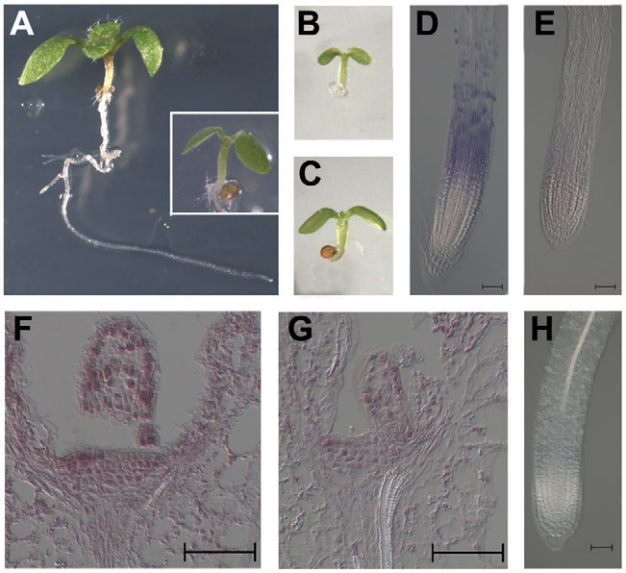

Fig. 1. The seedling phenotype of cytokinin triple-receptor mutants. (**A-C**) Eight-day-old *Col-0* (A), *ahk2-2 ahk3-3 cre1-12* (B), and *stip-1* (C) seedlings grown with no exogenous sucrose. The inset in (A) shows a two-day-old *Col-0* seedling at the same magnification. (**D**,**E**,**H**) Wholemount in situ hybridization with an anti-sense *STIP* probe in two-day-old *Col-0* (D), *ahk2-2 ahk3-3 cre1-12* (E), and the type-B *arr1-3 arr10-5 arr12-1* (H) roots. The signal is nearly absent in E and H. (**F**,**G**) In situ hybridization with an anti-sense *STIP* probe on longitudinal sections through the center of the shoot apical meristems of two-day-old *Col-0* (F) and *ahk2-2 ahk3-3 cre1-12* (G) seedlings. The signal is reduced in G. Scale bars: 50 μm.

promote meristem growth. We therefore examined the phenotype of the cytokinin triple receptor mutants without exogenously supplied sugar. One week after germinating on sugarless media, the wild-type seedlings had already produced true leaves and lateral roots (Fig. 1A). By contrast, the *ahk2-2 ahk3-3 cre1-12* (Higuchi et al., 2004) seedlings were still similar in size to two-day-old wild-type seedlings and the cotyledons failed to fully expand and turn green (Fig. 1B, compare with 1A inset). This phenotype indicates that cytokinin signaling is essential for early seedling development, and is reminiscent of that of the *stip* mutants, which enter complete growth arrest following germination without exogenous sugar (Fig. 1C) (Wu et al., 2005). The growth-arrested *ahk2-2 ahk3-3 cre1-12* seedlings could resume limited growth when transferred to sucrose-containing media, producing the phenotypes described previously (data not shown) (Higuchi et al., 2004; Nishimura et al., 2004).

STIP and cytokinin pathways share downstream components

The similarity of *stip* and *ahk2-2 ahk3-3 cre1-12* seedling phenotypes led us to examine the relationship between *STIP* and cytokinin signaling. To identify molecular events downstream of *STIP*, we generated transgenic plants (XW119) in which *STIP* expression throughout the plant was under the control of the ethanol-inducible *alc* system (Caddick et al., 1998; Roslan et al., 2001; Salter et al., 1998). After 1 hour of treatment with 2% ethanol, the *STIP* expression level increased by 73-fold. When we examined the mRNA profile of these plants with ATH1 expression arrays, we found that approximately 800 genes were differentially regulated compared with those of the uninduced plants (P<0.01).

Previously, in an effort to characterize the cytokinin signaling pathway, Brenner and colleagues (Brenner et al., 2005) expression-profiled wild-type seedlings treated with 5 μM benzyladenine (BA), a cytokinin analog, for 15 and 120 minutes. Using the same criteria as for the XW119 arrays, we identified genes responsive to BA treatments in their experiment. Of the 109 genes that were differentially expressed after the 15 minute BA treatment, 31 were also affected by elevated *STIP* expression (P=0.00087 based on the hypergeometric test), including 13 transcription factor genes (Table 1). Unfortunately, most of these genes have not been studied for their involvement in meristem development. Furthermore, 107 of the 475 genes that were affected after the 120 minute BA treatment were also found to respond to *STIP* induction (P=2.6e-54, based on the hypergeometric test; see Table S1 in the supplementary material), suggesting partial overlaps between the *STIP* and cytokinin pathways.

Cytokinin sensing mutants have reduced *STIP* expression

As sucrose can restore *stip* mutant seedlings to normal growth (Wu et al., 2005), but fails to completely rescue the *ahk2-2 ahk3-3 cre1-12* mutants (Higuchi et al., 2004; Nishimura et al., 2004), we hypothesized that *STIP* acts downstream of cytokinin perception during seedling development, and that part of the cytokinin triple-receptor mutant phenotype might be due to the lack of *STIP* activity. To test this model, we examined *STIP* expression in two-day-old wild-type and *ahk2-2 ahk3-3 cre1-12* seedlings using in situ hybridization. In wild-type seedlings, *STIP* RNA was detected in the shoot apical meristem (SAM), the emerging leaf (Fig. 1F) and the upper root meristematic zone (Fig. 1D). By contrast, although the cytokinin receptor mutants are morphologically similar to the wild type at this stage, *STIP* expression was much reduced in their shoot apex (Fig. 1G) and there was very little detectable *STIP* expression in the roots (Fig. 1E). We also found similar reductions in *STIP* mRNA levels in the roots of the type-B *arr1-3 arr10-5 arr12-1* triple-mutant seedlings (Fig. 1H), which are nearly completely insensitive to cytokinin stimulation in the roots (Argyros et al., 2008; Yokoyama et al., 2007). Although we cannot rule out the possibility that the observed reductions in *STIP* expression are caused by morphological changes due to the lack of cytokinin signaling, these findings are consistent with our hypothesis that *STIP* acts downstream of the cytokinin signaling pathways.

stip mutant seedlings show reduced cytokinin sensitivity in shoot growth

To further test the genetic relationship between *STIP* and cytokinin signaling, we examined the cytokinin sensitivity of *stip* mutants. Exogenous cytokinin normally inhibits seedling growth. The wild-type shoots stopped developing after being transferred to media containing 100 nM trans-zeatin, with the cotyledons and the existing leaves accumulating anthocyanin pigment, an indicator of stress response (Fig. 2C). This became even more apparent on higher concentrations of zeatin (Fig. 2D). By contrast, *stip-1* seedlings continued to develop in response to the sucrose in the media, and had noticeably less anthocyanin in their leaves (Fig. 2G,H). Similar results were obtained using cytokinin N^6-(Δ^2-isopentenyl)-adenine (2-iP; see Fig. S1 in the supplementary material). However, *stip-1* showed wild-type responses to cytokinins in root inhibition, callus greening, and senescence assays in the presence of sucrose. In additional experiments, *stip* mutants showed normal responses to other hormones, including auxin, gibberellins, brassinosteroids and

Table 1. Genes affected by both 1 hour of *STIP* induction and 15 minute BA treatment

Gene	Fold change BA 15 minutes*	Fold change *STIP* 1 hour*	Predicted function
At1g03850	0.2571	2.6016	Monothiol glutaredoxin-S13
At1g13300	0.3147	2.2482	Myb transcription factor
At2g42280	0.3121	0.5128	bHLH transcription factor
At4g27410	0.3533	5.169	NAC transcription factor
At2g39980	0.3663	4.6685	Transferase family
At1g49500	0.428	2.6386	Unknown
At3g57040	0.3556	4.0815	ARR9
At3g61060	0.4381	3.9053	F-box protein PP2-A13
At1g21830	0.4463	2.9853	Unknown
At5g47060	0.5069	0.4704	Senescence-associated protein-related
At1g68670	0.514	5.2182	Myb transcription factor
At1g21670	0.4901	0.5296	Unknown
At5g01740	2.8546	0.2073	Wound-responsive protein-related
At1g23030	2.7884	1.812	Armadillo/beta-catenin repeat protein
At1g55960	4.0683	2.0493	Unknown
At5g61600	2.7033	2.7744	B-3 type ERF/AP2 transcription factor
At4g28250	2.0912	1.915	Beta-expansin/allergen protein
At4g01250	4.5594	2.2372	WRKY transcription factor; group II-e
At3g55980	4.4222	3.4661	CCCH-zinc finger transcription factor
At4g16515	2.1842	2.016	Unknown
At3g47340	3.3162	0.3483	Dark-inducible 6
At4g08950	2.0534	0.4789	Phosphate-responsive protein
At4g32190	2.2607	2.4067	Centromeric protein-related
At5g48430	3.0643	0.5751	Aspartic-type endopeptidase/pepsin A
At2g21210	1.6912	1.9574	Respond to auxin stimulus and chitin
At5g67300	1.7858	2.3842	Myb transcription factor, MYB44
At3g23030	2.9137	2.3055	Transcription factor, IAA2
At3g47500	2.1958	0.5685	Dof-type zinc finger transcription factor
At3g59060	3.1462	2.0658	PIF5
At5g48490	2.4646	4.1967	Protease inhibitor/lipid transfer protein
At3g11670	2.1356	2.5456	Assembly of galactolipids

*Calculated as: expression level before treatment/expression level after treatment.

abscisic acid, when grown on sucrose-containing media. These results indicate that *STIP* acts downstream of cytokinin perception during early seedling development.

STIP activates the expression of a subset of cytokinin response factors

At the molecular level, a plant's ability to respond to cytokinin stimulation can be measured by the expression of the immediate cytokinin response genes, a group of type-A *ARR* genes including *ARR5*. To further analyze the role for *STIP* in cytokinin signaling, we compared *ARR5* expression patterns in wild-type and *stip* mutant seedlings using in situ hybridization. It has been previously reported that, in five-day-old seedlings, *ARR5* is expressed weakly in the SAM, and stronger expression can be detected in the root meristem region (D'Agostino et al., 2000). We detected a similar *ARR5* mRNA pattern in two-day-old wild-type seedlings (Fig. 3A,C). In comparison, *ARR5* expression levels were much reduced in both the shoot and the root of *stip-1* mutants (Fig. 3B,D). We confirmed the reduction of *ARR5* expression in the shoot apex using a β-glucuronidase (GUS) reporter under the control of a 1.6 Kb *ARR5* promoter, which has been shown to respond to cytokinin induction (D'Agostino et al., 2000). Two days after germination, strong ARR5::GUS activity could be detected in the SAM of the wild-type seedlings (Fig. 3E) and was significantly reduced in *stip-2* seedlings (Fig. 3F).

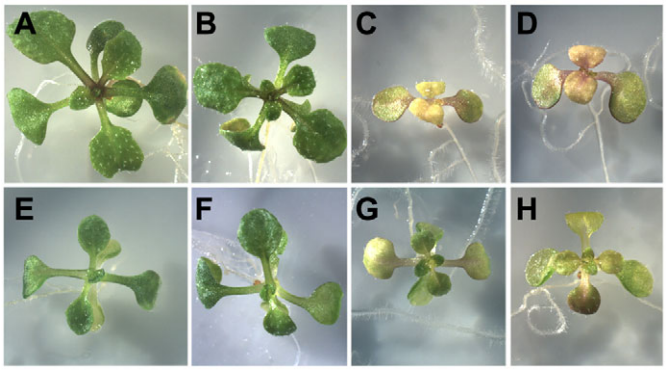

Fig. 2. *stip* mutants have reduced sensitivity to cytokinin inhibition. (**A-H**) Seven days after *Col-0* (A-D) and *stip-1* (E-H) seedlings were transferred to media containing sucrose and 0 (A,E), 10 nM (B,F), 100 nM (C,G), and 1 μM (D,H) of trans-zeatin, *stip* seedlings show continued growth and reduced anthocynin accumulation (G,H).

We also compared the expression levels of additional cytokinin signaling components between two-day-old wild-type and *stip-1* seedlings, using reverse transcription followed by real-time PCR analysis (qRT-PCR). In this experiment, we included three other type-A *ARR* genes (*ARR6*, *7* and *9*), three type-B *ARR* genes (*ARR1*, *10* and *12*), and two *CRF* genes (*CRF2* and *CRF5*), which are involved in cytokinin signaling during early seedling development (Rashotte et al., 2006). Both of the type-A *ARR* genes and the *CRF* genes tested showed significant reduction in the *stip-1* background, with *CRF2* being most strongly affected (Table 2; Fig. 4A), indicating that *STIP* is involved in their transcriptional activation. As the two-day-old *stip-1* seedlings are morphologically similar to the wild-type, and still have cell cycle activities in both meristems (Wu et al., 2005), we believe that the observed changes in gene

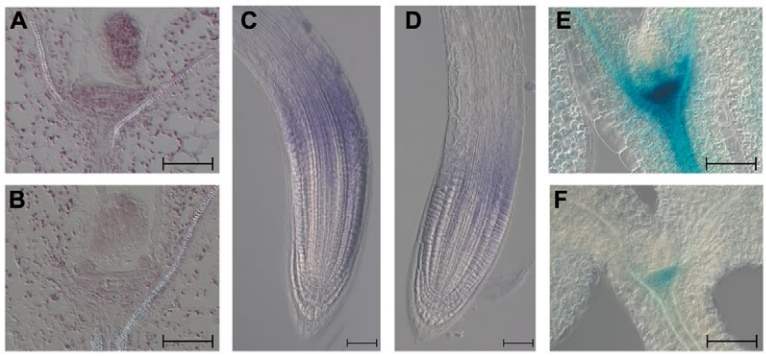

Fig. 3. *ARR5* (type-A *ARR*) expression is reduced in *stip* mutants. (**A**,**B**) In situ hybridization with an anti-sense *ARR5* probe on longitudinal sections through the center of the shoot apical meristems of two-day-old *Col-0* (A) and *stip-1* (B) seedlings. The signal is reduced in B. (**C**,**D**) Wholemount in situ hybridization with an anti-sense *ARR5* probe in two-day-old *Col-0* (C) and *stip-1* (D) roots. The signal is reduced in D. (**E**,**F**) ARR5::GUS activity staining in two-day-old *Col-0* (E) and *stip-2* (F) seedling shoots. GUS activity is reduced in F. Scale bars: 50 μm.

expression profiles in *stip* mutants are caused by the lack of *STIP* activities, instead of resulting indirectly from the onset of the seedling growth arrest. By contrast, we found no significant change in the expression levels of the type-B *ARR1*, *10* and *12*, in *stip* mutant seedlings (Table 2), suggesting that *STIP* does not affect type-B *ARR* transcription.

The above findings suggest that *STIP* acts upstream of the type-A *ARR* and the *CRF* genes, all of which are also transcriptionally activated by the type-B ARR proteins. This result can be explain by one of the two following mechanisms: a linear pathway where *STIP* is required for type-B *ARR* function in activating these genes, or *STIP* acts in parallel to the type-B *ARRs* in this process. To distinguish these two possibilities, we analyzed the ability of cytokinins to induce the expression of the above-mentioned target genes, which is known to require type-B ARR protein activities, in *stip* mutants. If the linear model was correct, we would expect to find reduced induction of these genes in *stip* mutants. In comparison, the parallel model would predict normal induction in *stip* background. As shown in Table 2, after 30 minutes of 250 nM zeatin treatment, both the type-A *ARR6*, *7*, *9* and *CRF2*, *5* genes showed a similar to or higher than wild-type level of induction in *stip-1*, suggesting that *STIP* acts in parallel to the type-B *ARRs* to activate these genes. However, consistent with our hypothesis that *STIP* plays a positive role in the activation of cytokinin response genes, the absolute post-induction transcript levels of four of the five genes remained below that of the wild type in *stip-1* (Fig. 4A). Only *ARR7* expression in *stip-1* after cytokinin induction reached similar levels as those in the wild type, which could be attributed to the reduced expression of its repressor *WUS* in *stip* mutants (Leibfried et al., 2005; Wu et al., 2005).

stip mutants show normal feedback regulation by type-A *ARR* genes

The type-A *ARR* genes modulate cytokinin response through a feedback mechanism, which results in the downregulation of the cytokinin primary response genes after a brief peak in their RNA levels (D'Agostino et al., 2000). To determine whether *STIP* is involved in this regulation, we examined the cytokinin induction profiles of two type-A *ARR* genes, *ARR6* and *ARR7*, in two-day-old wild-type and *stip-1* seedlings over the course of three hours. Consistent with the previous report (D'Agostino et al., 2000), both *ARR6* and *ARR7* expression peaked after 15 minutes of induction in wild type, and both were downregulated after about 30 minutes. *stip-1* seedlings showed a more dramatic initial induction of *ARR6* and *ARR7*. Their expression levels continued to increase until 30 minutes of treatment, after which both were downregulated, in a manner similar to what was seen in the wild type. In addition, *ARR9*, another type-A *ARR* gene, displayed similar induction patterns between the wild-type and *stip-1* samples (Fig. 4B). These observations indicate that the loss of *STIP* activities does not interfere with the negative feedback by the type-A *ARR* genes. The slight shift observed in *ARR6* and *ARR7* induction profiles might be owing to the fact that both genes were expressed at much lower levels in *stip-1* prior to zeatin treatment, and therefore required a longer time to reach the threshold for triggering the negative feedback responsible for attenuating the induction responses.

STIP over-expression partially restores SAMs in cytokinin sensing mutants

To assess how much the lack of *STIP* contributes to the seedling arrest phenotype of the cytokinin triple-receptor mutants, we over-expressed *STIP* in a *ahk2-2 ahk3-3 cre1-12* background, taking advantage of the activation-tagged *stip-D* allele (Wu et al., 2005). As *STIP* expression in this context is driven by the 35S enhancers, it bypasses the requirement for cytokinin signaling in *STIP* activation. Two weeks after germination, the *ahk2-2 ahk3-3 cre1-12* seedlings that also express an activation-tagged *STIP* (*pAS1*) were fully green and continued to develop without exogenous sugar (Fig. 5C), although they grew much slower than the wild type and the leaves did not fully expand (compare with Fig. 5A). A limited root system was also able to develop in *ahk2-2 ahk3-3 cre1-12* in response to *pAS1*. Phenotypically, these partially rescued seedlings resembled the cytokinin receptor mutants germinated on sucrose-containing media (Fig. 5D) (Higuchi et al., 2004; Nishimura et al., 2004).

The partial rescue by *pAS1* is reflected in the size of the SAM. At this stage, the wild-type SAM has reached its mature size and transitioned from vegetative to inflorescence meristem (Fig. 5E). Consistent with its arrested growth, the SAM in *ahk2-2 ahk3-3 cre1-12* seedlings failed to develop after germination and remained flat

Table 2. Relative gene expression levels in response to 30 minutes of induction by 250 nM trans-zeatin

	CRF2	*CRF5*	*ARR6* (A)	*ARR7* (A)	*ARR9* (A)	*ARR12* (B)
stip-1/*Col-0*	0.18	0.59	0.37	0.31	0.46	0.82
Col-0 induced/*Col-0*	1.66	1.45	3.94	4.33	1.38	ND
stip-1 induced/*stip-1*	4.25	1.64	5.61	16.8	1.86	ND

The letters in parentheses indicate the class of the *ARR* genes. ND, not determined.

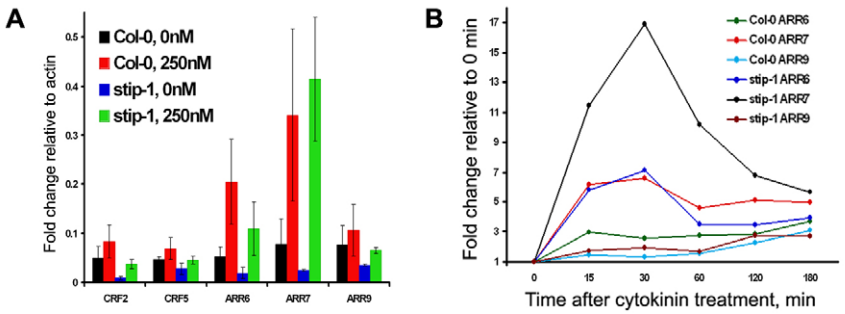

Fig. 4. Relative expression levels of selected cytokinin target genes in *stip* mutants. (**A**) Two-day-old seedlings were treated with 250 nM trans-zeatin or mock for 30 minutes and gene expression levels were measured by qRT-PCR. All samples were normalized to *ACT8*. *ARR6*, *7* and *9* are all type-A *ARR* genes. (**B**) Two-day-old seedlings were treated with 250 nM trans-zeatin for a total of 3 hours and gene expression levels were measured by qRT-PCR at different timepoints. After normalizing to *ACT8*, each gene is normalized to its pre-induction levels in the appropriate genotype.

(Fig. 5F). In comparison, the *pAS1*-rescued cytokinin triple-receptor mutants developed a dome-shaped SAM, although it was smaller than in the wild type (Fig. 5E,G). This finding strongly supports the notion that *STIP* mediates cytokinin signaling in promoting SAM growth. Consistent with our earlier finding that sucrose acts downstream of *STIP* in promoting meristem development (Wu et al., 2005), the sucrose-rescued *ahk2-2 ahk3-3 cre1-12* mutants had a flatter SAM compared with that of the *pAS1*-rescued plants (Fig. 5H).

As *pAS1* was also able to stimulate limited root growth in the cytokinin receptor mutants, we examined its effect on the root meristems in these seedlings. Without cytokinin perception, the roots of *ahk2-2 ahk3-3 cre1-12* seedlings fail to develop. A closer examination revealed that the *ahk2-2 ahk3-3 cre1-12* root morphology was largely normal, with the exception that the root meristematic zone was much reduced compared with that of the wild type (Fig. 5I,J). Interestingly, increased *STIP* expression driven by *pAS1* not only failed to improve, but also led to slight reductions in the size of the root meristematic zones in *ahk2-2 ahk3-3 cre1-12* (Fig. 5K). A similar phenotype was seen in the roots of the sugar-grown *ahk2-2 ahk3-3 cre1-12* seedlings (Fig. 5L). Both the changes in root cellular morphologies and the length of the root vasculatures point to earlier differentiation of the meristematic cells in the *pAS1 ahk2-2 ahk3-3 cre1-12* roots (Fig. 5K), which is similar to the result of exogenous cytokinin application (Dello Ioio et al., 2007).

Ectopic *STIP* expression differentially regulates cytokinin target genes in cytokinin perception mutants

We next compared the expression levels of selected cytokinin and *STIP* common target genes in two-day-old cytokinin perception mutants with or without *pAS1* rescue. For this experiment, we included *ARR6*, *ARR7*, both type-A *ARR* genes, *CRF2*, and two of the transcription factors identified as potential common downstream factors between *STIP* and cytokinin induction in our microarray analysis, *At4g27410* and *At1g68670* (Table 1; see also Table S1 in the supplementary material). As shown in Table 3 and Fig. 6, all six genes were expressed at lower levels in *ahk2-2 ahk3-3 cre1-12* mutants than in wild-type seedlings. In *pAS1 ahk2-2 ahk3-3 cre1-12*, increased *STIP* levels affected these genes in different manners. Both *ARR6* and *ARR7* were further repressed, possibly owing to an overactive negative-feedback loop. Consistent with the requirement for the type-B ARRs in *CRF2* activation (Rashotte et al., 2006), *pAS1* was only able to partially rescue *CRF2* expression. Conversely, *pAS1* restored *At4g27410* and *At1g68670* expression to the wild-type levels. These results strongly support our hypothesis that *pAS1* partially rescues the cytokinin triple-receptor mutants by activating components of the cytokinin signaling network.

DISCUSSION

Although specified during embryogenesis, primary meristems only become fully established during early vegetative growth, which requires *STIP* activity. We have shown that cytokinin signaling is essential for this process, and that it does so partly through the function of *STIP*.

Cytokinin signaling is essential for seedling and meristem development

Based on results from tissue culture experiments, it has been proposed that cytokinin signaling is essential in meristem establishment and size regulation (Mok and Mok, 2001). However, previous reports showed that loss of cytokinin perception only results in the reduction of meristem size and seedling growth rate (Higuchi et al., 2004; Nishimura et al., 2004). We have found that the phenotypic severity of the cytokinin receptor mutant *ahk2-2*

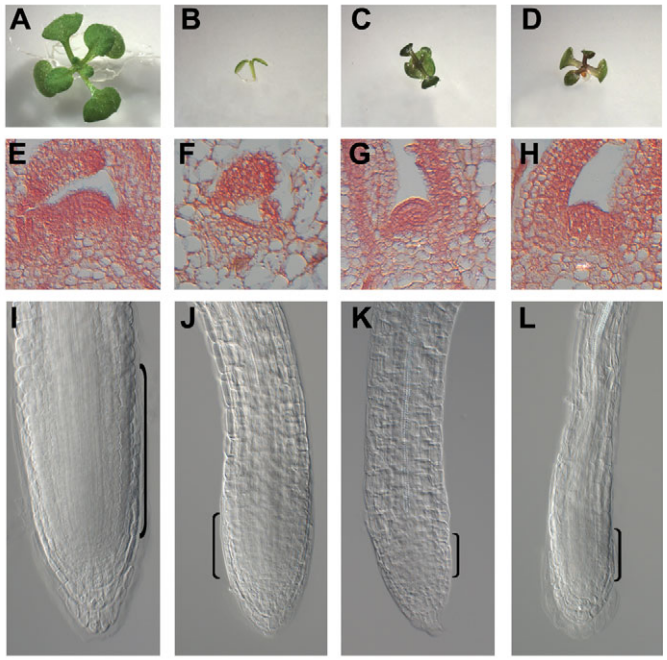

Fig. 5. *STIP* over-expression partially rescues the cytokinin triple-receptor mutants. (**A-D**) 12-day-old *Col-0* (A), *ahk2-2 ahk3-3 cre1-12* (B), *pAS1 ahk2-2 ahk3-3 cre1-12* (C) seedlings grown without supplemented sugar, and (D) *ahk2-2 ahk3-3 cre1-12* of the same age grown on sucrose-containing media. (**E-H**) Longitudinal sections through the center of the shoot apices of the seedlings shown in (A-D), stained with Safranin O. (**I-L**) DIC images of cleared root tips of the seedlings shown in (A-D). The positions of the root meristematic zones are marked with brackets.

Table 3. Relative gene expression levels in response to *pAS1* rescue

	ARR6 (A)	*ARR7* (A)	*CRF2*	*At4g27410*	*At1g68670*
ahk2-2 ahk3-3 cre1-12/Col-0	0.17	0.21	0.29	0.62	0.53
pAS1 ahk2-2 ahk3-3 cre1-12/Col-0	0.05	0.04	0.49	1.07	0.92

The letters in parentheses indicate the class of the *ARR* genes.

ahk3-3 cre1-12 is affected by exogenous sucrose in the growth media. Without supplemented sugar, the cytokinin perception mutants completely fail to initiate growth after germination (Fig. 1B), indicating a lack of active meristems (Fig. 5F,J). In addition to their inability to initiate new organs, the cotyledons in *ahk2-2 ahk3-3 cre1-12* mutants fail to fully green and expand (Fig. 1B). A similar cotyledon phenotype has been reported in plants carrying mutations in the *CRF* genes, which are regulated by cytokinin stimulation at both the transcription and protein localization levels (Rashotte et al., 2006). These new findings underscore the importance of cytokinin signaling in seedling development and provide in vivo evidence that cytokinins are essential for the establishment of both the shoot and root meristems after germination.

STIP mediates a subset of cytokinin signaling events in seedling development

Previously, we showed that the homeodomain transcription factor gene *STIP* is essential for maintaining the meristematic fate and proliferation through *Arabidopsis* development (Wu et al., 2005). The striking similarities between *stip* and *ahk2-2 ahk3-3 cre1-12* seedling phenotypes (Fig. 1B,C) and the common downstream targets (Table 1) suggested a possible link between *STIP* and the cytokinin signaling network. Several lines of evidence place *STIP* downstream of cytokinin perception: the *ahk2-2 ahk3-3 cre1-12* and the type-B *arr1-3 arr10-5 arr12-1* seedlings have significantly lower levels of *STIP* expression (Fig. 1E,G,H); exogenous sucrose can fully rescue *stip* seedlings (Wu et al., 2005), but it is only able to partially restore the development of cytokinin triple-receptor mutants (Fig. 5D,H); *stip* mutants show reduced cytokinin sensitivity in selected assays (Fig. 2); *STIP* is involved in the activation of several known cytokinin signaling components (Fig. 3; Fig. 4A); and most importantly, ectopic *STIP* expression leads to the partial establishment of the shoot meristem and continued growth in cytokinin receptor mutants (Fig. 5C,G). However, it is worth noting that unlike the B-type *ARR* genes (Mason et al., 2005), *STIP* is only involved in limited aspects of cytokinin signaling, namely cotyledon and meristem establishment after germination, and that *STIP* also functions in additional pathways at later stages of development.

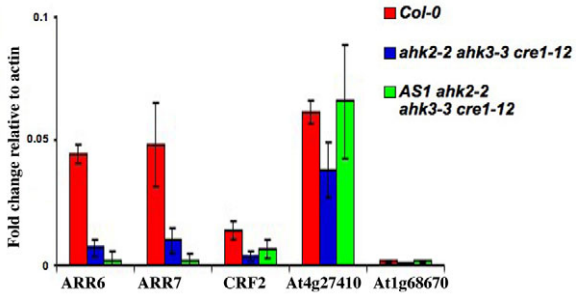

Fig. 6. Relative expression levels of selected cytokinin and STIMPY common target genes in mutant and *pAS1*-rescued *ahk2-2 ahk3-2 cre1-12* seedlings. Gene expression levels in two-day-old seedlings were measured by qRT-PCR and all samples were normalized to *ACT8*.

The cytokinin and auxin signaling networks interact extensively in modulating plant growth (Moubayidin et al., 2009). In early embryos, *STIP* might act partially redundantly with its closest homolog in *Arabidopsis*, *STIMPY-LIKE* (*STPL* or *WOX8*), in modulating auxin distribution (Breuninger et al., 2008). Therefore, it is formally possible that the cytokinin insensitivity of *stip* mutants results from disrupted auxin transport or signaling. However, experimental evidence argues against this mechanism: the DR5::GFP reporter (Friml et al., 2003) is expressed in the wild-type pattern and intensity in *stip* mutant embryos (Wu et al., 2007) and seedlings (data not shown), indicating normal auxin biosynthesis and localization; when treated with exogenous auxin on sugarless media, *stip* mutant roots display wild-type levels of auxin uptake; and once rescued by sucrose, the *stip* mutants display normal responses to auxin.

Cytokinin signaling regulates *STIP* expression

The much-reduced *STIP* expression in the cytokinin triple-receptor mutants and the type-B *arr1*, *10*, *12* triple mutants (Fig. 1) suggests that cytokinin signals are involved in *STIP* transcriptional activation. However, we did not detect an immediate increase in *STIP* transcription upon exogenous cytokinin induction using qRT-PCR, suggesting that *STIP* might not be a direct transcriptional target of the type-B ARR proteins. An additional layer of *STIP* regulation by cytokinin signals might occur post-translationally. STIP protein contains a relatively high percentage of serines and threonines. Similar to the CRF proteins (Rashotte et al., 2006), a functional STIP:GFP fusion protein expressed under the native *STIP* promoter

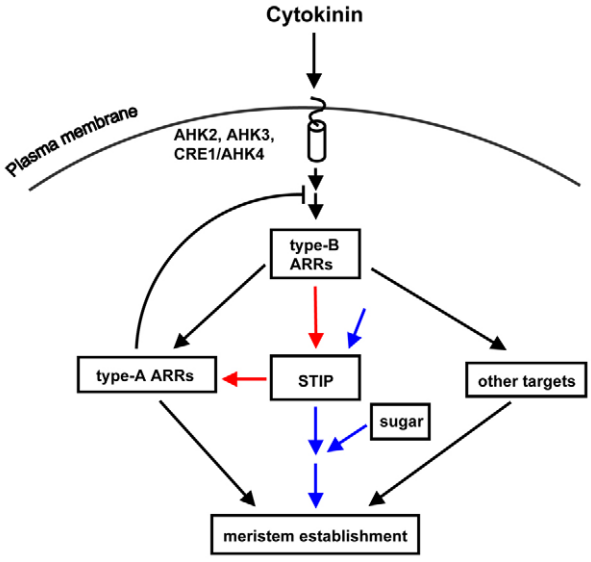

Fig. 7. A diagram of the proposed involvement of *STIP* in cytokinin signaling during vegetative SAM development. *STIP* activity is positively regulated by cytokinin stimulation, downstream of the type-B *ARR* gene functions. Together with other branches of the cytokinin signaling network, *STIP* acts upstream of sugar sensing/signaling in promoting meristem establishment.

is both nuclear and cytoplasmic (Wu et al., 2007), raising the possibility that STIP protein activity is modulated by phosphorylation. We are currently in the process of studying the mechanisms by which cytokinin signaling affects *STIP* expression and activity.

STIP plays different roles in the shoot and root meristems

Based on our findings, we propose that *STIP* acts in parallel to the previously studied cytokinin signaling pathway downstream of the type-B ARR proteins during the establishment of the shoot apical meristem. This hypothesis is supported by our finding that ectopic *STIP* can restore *At4g27410* and *At1g68670* expression to the wild-type levels in the cytokinin receptor mutants, bypassing the need for activated type-B ARR proteins (Fig. 6). And as the slightly smaller SAM in *pAS1 ahk2-2 ahk3-3 cre1-12* seedlings cannot account for the reduced growth rate and leaf size (Fig. 5C,G), it is probable that a combination of *STIP* and the previously known cytokinin signaling targets is required for mediating the full range of cytokinin responses during vegetative development (Fig. 7).

The root meristem responds to changes in cytokinin levels in a more complex fashion than the SAM. Although the cytokinin triple-receptor mutants develop root meristem arrest (Fig. 5J; Higuchi et al., 2004), increased root meristem activities have been observed in *ahk2 ahk3* double mutants (Riefler et al., 2006) and seedlings with increased cytokinin degradation (Werner et al., 2003). Interestingly, whereas *STIP* is essential in root meristem maintenance in plants with an otherwise intact cytokinin signaling pathway (Wu et al., 2005), ectopic *STIP* not only failed to rescue, but also resulted in further reductions of, the root meristems in the *ahk2-2 ahk3-3 cre1-12* triple mutants (Fig. 5K), mimicking the effect of exogenous cytokinin treatment (Dello Ioio et al., 2007). One possible explanation for this phenotype is that the elevated *STIP* levels in these mutants led to higher cytokinin signaling that is sufficient to inhibit root meristem. Another possibility is that a balance of activities between *STIP* and other cytokinin effectors is required for the proper development of the root meristem, as the *pAS1* transgene did not cause any visible root defect in wild-type or *ahk2-2 ahk3-3/+ cre1-12* seedlings. The precise involvement of *STIP* in cytokinin signaling during root meristem establishment remains to be investigated.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

Supplementary material for this article is available at
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Table S1. Genes affected by both 1 hour of *STIP* induction and 120 minute BA treatment

Gene	Fold change BA 120 minutes*	Fold change *STIP* 1 hour*	Gene	Fold change BA 120 minutes*	Fold change *STIP* 1 hour*
At3g47340	0.0274	0.3483	*At5g56100*	0.2643	4.9367
At3g48360	0.0648	2.9678	*At5g28770*	0.2685	2.4672
At3g61060	0.0836	3.9053	*At3g19030*	0.2663	2.989
At1g80440	0.1003	3.7259	*At5g66985*	0.2698	0.391
At1g03610	0.1015	3.0905	*At3g30775*	0.2736	0.3917
At1g21830	0.1019	2.9853	*At5g64920*	0.2743	0.531
At1g10140	0.1072	1.9124	*At4g27410*	0.2768	5.169
At4g27260	0.1161	1.8145	*At1g21670*	0.2798	0.5296
At5g12050	0.1183	3.1192	*At5g57660*	0.2888	1.934
At4g29190	0.1184	6.028	*At1g75180*	0.2969	2.113
At2g20670	0.12	5.1837	*At4g28240*	0.2966	2.8457
At2g18700	0.1221	3.3074	*At2g34510*	0.2997	0.4976
At5g47980	0.1262	2.7688	*At5g62280*	0.3023	2.8538
At5g01740	0.1451	0.2073	*At3g01400*	0.2995	0.4596
At5g19120	0.1477	3.6779	*At2g17450*	0.3041	2.2688
At2g39980	0.153	4.6685	*At4g28270*	0.3029	3.417
At5g60860	0.1521	0.5347	*At3g26510*	0.3092	4.4381
At3g17510	0.1528	2.2096	*At2g26980*	0.3149	2.8043
At2g25900	0.1592	6.8034	*At1g72180*	0.3135	0.5651
At4g02440	0.1623	0.5156	*At4g17460*	0.3189	1.8094
At1g70290	0.1647	4.2322	*At3g61980*	0.3212	0.4981
At1g60010	0.1637	1.745	*At4g21990*	31.7357	2.6212
At4g03510	0.1703	2.9617	*At5g48430*	30.2558	0.5751
At1g68670	0.1708	5.2182	*At4g04610*	22.1133	3.4548
At5g16030	0.174	3.3382	*At2g38940*	16.8409	1.846
At3g57040	0.1792	4.0815	*At5g49080*	15.8265	1.9944
At3g47295	0.1815	2.1136	*At5g57530*	14.5378	2.3307
At4g35750	0.1844	1.7412	*At5g08640*	14.6609	2.0373
At2g42280	0.1785	0.5128	*At3g58990*	12.5812	1.9194
At4g16515	0.188	2.016	*At3g52370*	12.0271	1.8454
At1g55810	0.1834	0.55	*At2g47140*	10.941	0.2816
At5g49450	0.1919	3.342	*At5g58900*	10.5452	0.5715
At5g58650	0.1951	0.5678	*At4g35060*	8.5373	1.6134
At3g13310	0.2009	0.557	*At5g03760*	8.3432	1.7063
At3g10020	0.2043	1.6136	*At3g43800*	8.3323	1.7433
At4g36040	0.2078	2.392	*At5g35190*	7.9026	1.5669
At1g10150	0.2093	2.4193	*At3g18000*	7.4532	2.1492
At2g40000	0.212	4.6563	*At1g30510*	7.507	1.7767
At1g32540	0.208	1.7775	*At4g28250*	7.6326	1.915
At3g59940	0.2122	3.4612	*At1g65840*	7.137	0.5502
At5g22270	0.2138	9.2645	*At2g31750*	6.6628	1.7472
At3g15630	0.2206	1.8899	*At1g51680*	6.2082	1.8741
At1g25560	0.2209	2.8929	*At3g62270*	6.0682	1.8203
At5g60680	0.2261	6.3016	*At3g07320*	5.8338	0.4502
At1g49500	0.2362	2.6386	*At1g55450*	5.7982	1.9612
At1g71970	0.2411	2.4178	*At4g21830*	5.653	0.5135
At3g06750	0.2408	2.1369	*At3g55120*	5.1002	1.7988
At5g22920	0.2513	2.1941	*At2g32210*	4.6074	0.4956
At1g56220	0.2548	2.255	*At1g79270*	4.5053	2.0054
At5g21170	0.2564	1.6547	*At2g17630*	4.469	0.2841
At4g13830	0.2583	1.8695	*At3g09940*	4.4566	0.4805
At4g05070	0.2596	3.6493	*At4g01660*	4.5213	0.433
At1g03850	0.2632	2.6016	*At3g23730*	4.4291	0.4543
At1g28330	0.2645	2.5136			

*Calculated as: expression level before treatment/expression level after treatment.