A Transcriptome-Based Characterization of Habituation in Plant Tissue Culture[1][W]

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For the last 50 years, scientists have recognized that varying ratios of the plant hormones cytokinin and auxin induce plant cells to form particular tissues: undifferentiated calli, shoot structures, root structures, or a whole plant. Proliferation of undifferentiated callus tissue, greening, and the formation of shoot structures are all cytokinin-dependent processes. Habituation refers to a naturally occurring phenomenon whereby callus cultures, upon continued passage, lose their requirement for cytokinin. Earlier studies of calli with a higher-than-normal cytokinin content indicate that overproduction of cytokinin by the culture tissues is a possible explanation for this acquired cytokinin independence. A transcriptome-based analysis of a well established habituated Arabidopsis (*Arabidopsis thaliana*) cell culture line was undertaken, to explore genome-wide expression changes underlying the phenomenon of habituation. Increased levels of expression of the cytokinin receptor *CRE1*, as well as altered levels of expression of several other genes involved in cytokinin signaling, indicated that naturally acquired deregulation of cytokinin-signaling components could play a previously unrecognized role in habituation. Up-regulation of several cytokinin oxidases, down-regulation of several known cytokinin-inducible genes, and a lack of regulation of the cytokinin synthases indicated that increases in hormone concentration may not be required for habituation. In addition, up-regulation of the homeodomain transcription factor *FWA*, transposon-related elements, and several DNA- and chromatin-modifying enzymes indicated that epigenetic changes contribute to the acquisition of cytokinin habituation.

Totipotency, i.e. the ability of a single cell to develop into a new organism, has been studied in plant cells for the last 50 years (Skoog and Miller, 1957; Steward et al., 1958; Murashige and Skoog, 1962). Totipotency involves two major developmental processes: dedifferentiation and redifferentiation. In plants, these processes are directed by the relative concentrations of the plant hormones cytokinin and auxin (Skoog and Miller, 1957; Steward, 1970), such that a high cytokinin to auxin ratio promotes shoot development, whereas a low cytokinin to auxin ratio promotes root development. Proliferation of dedifferentiated plant cell cultures (calli), greening, and the formation of shoot structures are cytokinin-dependent processes. Habituation is a naturally occurring phenomenon in which the division and growth of plant cell cultures, upon continued passage, eventually become independent of this requirement for cytokinin (Murashige and Skoog, 1962; Boeken et al., 1974; Meins, 1989).

Habituation is a mitotically transmissible character (Meins, 1989, and refs. therein). That is, new callus cultures derived from habituated tissues retain the cytokinin-independent state. To date, several loci (*Habituated leaf* [*Hl*]) conferring habituation on tobacco (*Nicotiana tabacum*) leaf tissues have been identified (*Hl-1*, *Hl-2*, and *Hl-3*), two of which were reported to be meiotically transmissible (Meins and Foster, 1986; Meins, 1989; Meins and Seldran, 1994; Meins and Thomas, 2003). Interestingly, this meiotic transmission of habituation is reversible (Meins and Thomas, 2003). One explanation for the reversibility of the heritable, habituated state is that the genetic alteration at the *Hl-2* locus, for example, is due to DNA modification rather than mutation. Indeed, variations in DNA methylation levels have been documented during the tissue culture process, as well as among plants regenerated from tissue culture and their progeny (Planckaert and Walbot, 1989; Phillips et al., 1994; Kaeppler et al., 2000; Fojtova et al., 2003; Hao et al., 2004; Koukalova et al., 2005).

Presently, experimental evidence for the mechanism of habituation is scant. Whereas ectopic expression of cytokinin-signaling components has been shown to artificially confer a habituated state on plant tissues (e.g. *CYTOKININ INDEPENDENT 1* [*CKI1*; Kakimoto, 1996], *ARABIDOPSIS RESPONSE REGULATOR 1* [*ARR1*; Sakai et al., 2001], *ARR2* [Hwang and Sheen, 2001], and *ARR4* [Osakabe et al., 2002]), overproduction of cytokinin by the plant tissues has been a predominant explanation for acquired cytokinin independence (Murashige and Skoog, 1962; Meins, 1989; Nogué et al., 2000; Catterou et al., 2002; Sun et al., 2003). One approach toward identifying the mechanism of habituation is a comparison of gene expression in

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habituated and nonhabituated cell cultures. If overproduction of cytokinin is the mechanism for habituation, then this could be reflected by increased expression of genes encoding cytokinin synthases (*IPT*s), decreased expression of genes encoding cytokinin-degrading enzymes (*CKX*s), and increased expression of known cytokinin-inducible genes (*KNAT1*, *CYCD3*, *CAB1*, *NIA1*, Type-A *ARR*s) in habituated calli. Likewise, if epigenetic modifications play a role in habituation, then this may be reflected by alterations in the expression levels of genes encoding enzymes involved in DNA methylation and/or histone modification.

This study explored the mechanism of habituation in the well established T87 Arabidopsis (*Arabidopsis thaliana*) cell culture line (Axelos et al., 1992). A transcriptome-based analysis of habituated and nonhabituated plant cell cultures revealed differential expression of more than 800 genes, which included a 19-fold up-regulation of the transcript encoding the cytokinin receptor *CRE1*. A concomitant overexpression of the CRE1 protein was verified using an isotope-assisted mass spectrometric method called AQUA (an acronym for absolute quantification of proteins) developed by Gerber et al. (2003). Phenotypic and transcriptomic differences between habituated and nonhabituated cell cultures, as well as potential mechanisms for the phenomenon of habituation, are discussed.

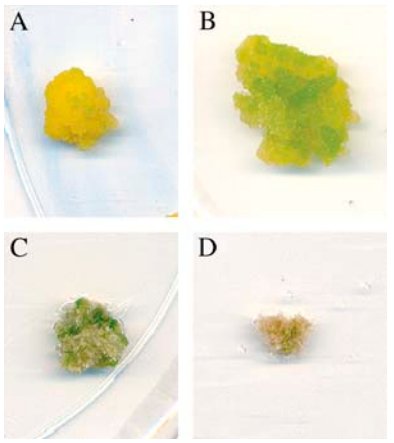

Figure 1. The effect of cytokinin on the growth and morphology of habituated and nonhabituated callus cultures. A, Representative habituated callus culture maintained in the presence of cytokinin (benzyl adenine) for 3 weeks. B, Representative habituated callus culture maintained in the absence of cytokinin for 3 weeks. C, Representative nonhabituated callus culture maintained in the presence of cytokinin for 6 weeks. D, Representative nonhabituated callus culture maintained in the absence of cytokinin for 6 weeks.

RESULTS

Phenotypic Differences between Habituated and Nonhabituated Callus Tissue

On solid media, callus tissue that has been freshly induced from Arabidopsis root segments (also called FC for freshly derived callus, or nonhabituated callus tissue) has an obvious requirement for exogenous cytokinin for maximal growth (Figs. 1 and 2). Habituated callus tissue derived over several passages onto solid media from the Arabidopsis T87 cell line (also T87), on the other hand, does not require cytokinin for maximal growth. In fact, proliferation of habituated callus tissues is inhibited by exogenously applied cytokinin (Figs. 1 and 2). T87 cell cultures are rapidly dividing with respect to freshly derived callus cultures and have a visibly different morphology. For example, cells within the T87 callus clumps are more easily teased apart and have a shiny appearance.

Transcriptome-Based Analysis of Habituated versus Nonhabituated Callus Tissue

Full-genome transcriptome analyses of habituated callus cultures grown in the presence or absence of cytokinin (T87 + BA, T87 − BA), as well as freshly derived (nonhabituated) callus cultures grown in the presence or absence of cytokinin (FC + BA, FC − BA), were carried out on the Arabidopsis 60mer microarray (NimbleGen Systems). Robust multiarray averaging and $\log_2$ transformation were applied to the data before analysis. A very strong positive correlation was seen for all possible pairwise comparisons of signal intensities across approximately 28,000 genes, within each set of four biological replicates, hereafter referred to as "groups" (T87 + BA, T87 − BA, FC + BA, and FC − BA; Table I), demonstrating the reproducibility of the technology.

Three of the six possible comparisons between groups were explored (Table II): (1) FC + BA versus FC − BA (to identify genes whose expression is directly or indirectly regulated by cytokinin in nonhabituated callus, and to compare this dataset with those generated by other studies exploring cytokinin regulation of gene expression), (2) T87 + BA versus T87 − BA (to characterize the response of a habituated cell line to cytokinin, and to look for similarities and differences in the responses of habituated and nonhabituated callus tissues to this phytohormone), and (3) T87 − BA versus FC + BA (to characterize the transcriptome of healthy, habituated callus cultures with reference to healthy, nonhabituated callus cultures). This last comparison is the one we felt would best reveal transcriptome-based differences underlying habituation.

A variety of methods for identifying differentially expressed genes between groups that vary by treatment type, treatment time, or tissue type have been described in the literature (summarized in Dräghici, 2003). Some of the most common include choosing an arbitrary fold-change cutoff (e.g. all genes whose expression changes by 2-fold or more between groups are called differentially expressed) or applying a statistical test, e.g. Student's *t* test, ANOVA, rank product statistics, or significance analysis of microarrays (SAM). Several of these methods were applied to the dataset. A comparison of the 2-fold-change cutoff, *t* test, ANOVA, and SAM revealed the SAM method to be the most conservative

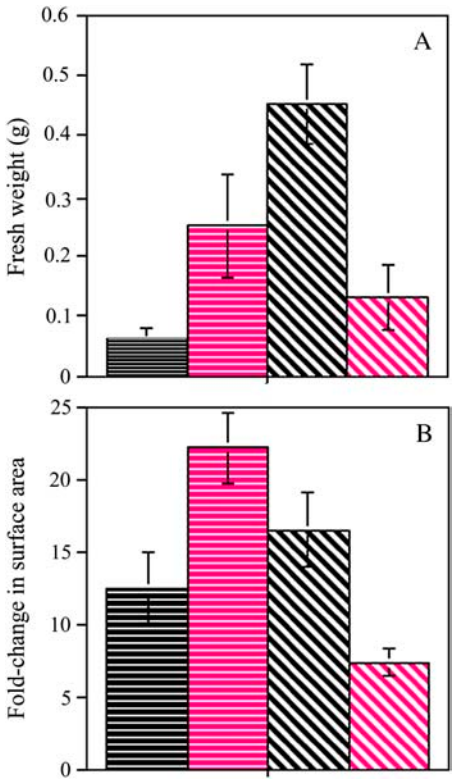

Figure 2. The effect of cytokinin on the proliferative capacities of habituated and nonhabituated callus cultures. Calli were cultured in the absence (black) or presence (magenta) of benzyl adenine. A, The fresh weight of callus cultures derived from nonhabituated (horizontal stripes) and habituated (diagonal stripes) tissues, after 6 and 3 weeks in culture, respectively. B, The fold-change in surface area of callus cultures derived from nonhabituated (horizontal stripes) and habituated (diagonal stripes) tissues, after 6 and 3 weeks in culture, respectively. At least 10 calli were measured for each data point. Error bars represent the SD.

for identifying differentially expressed genes between groups in this study (Table III).

A Comparison between the Groups FC + BA and FC − BA

For subsequent characterization of differential gene expression in this study, we chose to focus on the genes identified as significantly differentially expressed by the SAM method (Tusher et al., 2001). A comparison between the groups FC + BA and FC − BA identified 467 up-regulated genes (Supplemental Table I) and 23 down-regulated genes (Supplemental Table II). None of the 23 genes identified as significantly down-regulated by cytokinin in this study has been identified as a cytokinin down-regulated gene in the previous studies we queried (Crowell et al., 1990; D'Agostino et al., 2000; Hoth et al., 2003; Rashotte et al., 2003; Yamada et al., 2004; Kiba et al., 2005; Rashotte et al., 2005). On the other hand, 24 of the genes identified as cytokinin up-regulated in this study have been identified as cytokinin up-regulated genes in other studies as well (Supplemental Table I). Previous studies have identified cytokinin-regulated genes after hormone application to seedlings or tissue at intervals ranging from 10 min to 24 h. The overlap of 24 genes between this study and several previous studies represents genes whose expression remains up-regulated in response to cytokinin over an extended time period, i.e. on the order of months rather than minutes or hours. Not surprisingly, the Type-A response regulators (RRs), which are rapidly and transiently induced by cytokinins (Brandstatter and Kieber, 1998; Imamura et al., 1998; D'Agostino et al., 2000), were not identified as differentially expressed in nonhabituated calli. The previously identified cytokinin-regulated genes also identified in this study include several enzymes involved in cell wall modification (pectinacetylesterase, expansin, glycosyl hydrolases, and a pectin methylesterase inhibitor), nutrient and carbon acquisition (sulfate transporter, nitrate reductase, Suc transporter, Rubisco subunit), and light harvesting (chlorophyll *a*/*b*-binding proteins; Supplemental Table I).

Although only 5% (24/467) of the genes found to be up-regulated in nonhabituated calli in response to cytokinin were previously identified as cytokinin up-regulated, there is a much greater overlap (39%, 182/467) in the types of gene families identified among the cytokinin-regulated transcript studies (Supplemental

Table I. *Comparison of biological replicates*

T87, Calli derived from the T87 habituated cell line; FC, freshly derived nonhabituated calli; BA, the cytokinin benzyl adenine.

Treatment	Chip 1[a]	Chip 2[a]	R^2 Value[b]
T87 + BA	25294	25296	0.9601
T87 + BA	25294	16933	0.8005
T87 + BA	25294	16931	0.9083
T87 + BA	25296	16933	0.8272
T87 + BA	25296	16931	0.9006
T87 + BA	16933	16931	0.8891
T87 − BA	16818	25329	0.8725
T87 − BA	16818	25270	0.8737
T87 − BA	16818	16829	0.9427
T87 − BA	25329	25270	0.9352
T87 − BA	25329	16829	0.9055
T87 − BA	25270	16829	0.8764
FC + BA	25341	25331	0.9684
FC + BA	25341	16932	0.9284
FC + BA	25341	16820	0.9427
FC + BA	25331	16932	0.9260
FC + BA	25331	16820	0.9424
FC + BA	16932	16820	0.9780
FC − BA	25620	25343	0.9244
FC − BA	25620	16864	0.8644
FC − BA	25620	16822	0.8394
FC − BA	25343	16864	0.9082
FC − BA	25343	16822	0.8552
FC − BA	16864	16822	0.9653

[a]Chip number designated by NimbleGen Systems, designating individual hybridizations to the full-genome Arabidopsis microarray. [b]R^2 values were calculated based in the $\log_2$[signal intensity] for each gene represented on the microarray.

Table II. *All possible comparisons between groups*

T87, Calli derived from the T87 habituated cell line; FC, freshly derived nonhabituated calli; BA, the cytokinin benzyl adenine.

Group 1[a]	Group 2[a]	R^2 Value
T87 − BA	T87 + BA	0.9614
T87 − BA	FC − BA	0.8004
T87 − BA	FC + BA	0.8148
T87 + BA	FC − BA	0.7911
T87 + BA	FC + BA	0.7747
FC + BA	FC − BA	0.9073

[a]A group is defined as a particular combination of tissue type (T87 or FC) and treatment type (+ or − BA).

Table I). Among the genes found up-regulated by cytokinin in nonhabituated calli were several genes involved in light harvesting and photosynthesis, cell wall modification, amino acid and protein synthesis and transport, and nutrient and carbon acquisition. These findings correlate well with several known biological roles for cytokinins in planta, including promotion of greening (Stetler and Laetsch, 1965; Daniell and Rebeiz, 1982), cell division (Miller et al., 1956), and protein synthesis (Maaß and Klämbt, 1977), and serving as a nutrient sink (Roitsch and Ehneß, 2000). There were also a number of genes identified that potentially highlight cross talk between cytokinin-signaling and other signaling pathways, including auxin transport and signaling (At4g29080, At2g01420, At1g19840, At2g46690, At2g39730, At1g22630, At2g33830, At1g67700), calcium signaling (At4g33000, At1g25230, At5g54130), brassinosteroid synthesis (At4g13290, At1g78490, At4g13770, At2g34490, At3g26330, At3g03470, At2g22330), GA_3 synthesis (At1g06640, At5g59530), and ethylene responses (At5g07580, At5g61590).

A Comparison between the Groups T87 + BA and T87 − BA

A comparison between the groups T87 + BA and T87 − BA identified zero up-regulated genes and 36 down-regulated genes (Supplemental Table III). There was no overlap between genes down-regulated in the FC ± BA comparison and the T87 ± BA comparison, indicating that T87 cells respond to the presence of cytokinin differently than nonhabituated cells. Previous studies have shown that T87 cells can respond to transient cytokinin application by up-regulation of cytokinin primary response genes, similar to wild-type Arabidopsis seedlings (Yamada et al., 2004). This study, however, shows that at the level of the transcriptome, T87 calli and nonhabituated calli respond very differently to the presence or absence of exogenous cytokinin in the plant culture media. In fact, 19% (7/36) of the genes significantly down-regulated in habituated calli, in response to cytokinin, were found to be up-regulated in nonhabituated calli, in response to cytokinin (Supplemental Table III). Furthermore, an additional 53% (19/36) of these genes belong to gene families, some members of which were also identified as cytokinin up-regulated in nonhabituated calli or in previous studies (Supplemental Table III). Thus, while T87 cells may transiently respond to cytokinin in a physiologically relevant fashion, in the long term habituated T87 cells seem to respond to cytokinin in a manner quite opposite to nonhabituated cells.

A Comparison between the Groups T87 − BA and FC + BA

A comparison between the groups T87 − BA and FC + BA identified 440 up-regulated genes (Supplemental Table IV) and 405 down-regulated genes (Supplemental Table V). Thirty-two genes previously identified as cytokinin up-regulated and 31 genes previously identified as cytokinin down-regulated were also identified as up- and down-regulated, respectively, in habituated cells maintained in the absence of cytokinin (Supplemental Tables IV and V). The up- and down-regulated genes identified by SAM for the comparisons FC + BA versus FC − BA and T87 − BA versus FC + BA were categorized by biological process, using the gene ontology tool available

Table III. *Breakdown of significantly differentially expressed genes by test*

Bold text highlights the number of differentially expressed genes between groups based on the most conservative method used, SAM. T87, Calli derived from the T87 habituated cell line; FC, freshly derived nonhabituated calli; BA, the cytokinin benzyl adenine.

Method	Gene Expression Category	FC + BA versus FC − BA	T87 + BA versus T87 − BA	T87 − BA versus FC + BA
2-fold-change cutoff	Up-regulated genes	3,074	567	4,147
	Down-regulated genes	2,373	1,314	3,728
	Total differentially expressed genes	5,447	1,881	7,875
SAM[a,b]	Significantly up-regulated genes	467	0	440
	Significantly down-regulated genes	23	36	405
	Total significantly differentially expressed genes	**490**	**36**	**845**
t test[a,c]	Total significantly differentially expressed genes	6,993	2,347	9,320
ANOVA[a,d]	Total significantly differentially expressed genes	11,285	11,285	11,285

[a]Test performed using TIGR MultiExperiment Viewer (http://www.tm4.org/mev.html). [b]A gene's expression was considered significantly different between groups if a SAM analysis identified the gene ≥5/11 times. [c]A gene's expression was considered significantly different between groups if a *t* test identified the gene ≥5/10 times. [d]A gene's expression was considered significantly different between groups if a single ANOVA run identified the gene.

on The Arabidopsis Information Resource (TAIR) Web site (http://www.arabidopsis.org/tools/bulk/go/index.jsp; Table IV). Several of the biological process categories, into which the 490 genes with altered expression in nonhabituated calli fall, reveal a bias toward either up- or down-regulation of gene expression. For example, genes whose products are involved in cell organization and biogenesis, electron transport or energy pathways, and transport are more likely to be up-regulated than down-regulated. Perhaps this tendency toward up-regulation of genes important for energy and nutrient acquisition, and cell biogenesis, reflects the actively growing and dividing nature of nonhabituated callus tissue in the presence of cytokinin. Differentially expressed genes involved in nucleic acid and protein metabolism tend to be up-regulated in healthy habituated calli with respect to healthy nonhabituated calli, while genes involved in responding to environmental stimuli tend to be down-regulated. These tendencies may reflect that the T87 cell line has been selected for rapid proliferation, independent of cell division-promoting substances. Interpreted in this light, genes important for generating the nucleic acids and proteins necessary for rapid growth would tend to be up-regulated, while genes involved in sensing external growth and division cues would tend to be down-regulated.

Among those genes found up-regulated in habituated calli are the cytokinin-responsive His kinase (HK) *CRE1* (At2g01830) and the putative osmosensing HK *AtHK1* (At2g17820). Both *CRE1* and *AtHK1* have been identified as cytokinin up-regulated in previous studies as well (Che et al., 2002; Rashotte et al., 2003). The cytokinin receptor *AHK3* (At1g27320), on the other hand, was identified as down-regulated in habituated callus cultures. It is possible that this antagonistic regulation of two different cytokinin receptors in habituated calli reflects a compensatory measure. Microarray studies with various knockout mutants within the CRE family of cytokinin receptors (CRE1, AHK2, AHK3) may shed more light on this possibility. In any case, the differential expression of two members of the cytokinin-receptor family in Arabidopsis warranted a further look at the expression levels of genes involved in the cytokinin-signaling pathway.

Genes Involved in Cytokinin Responses

Cytokinin signaling in Arabidopsis occurs through a multistep His-to-Asp (His-Asp) phosphorelay (for review, see Hutchison and Kieber, 2002; Kakimoto, 2003). The key components of this signal transduction system are the receptor HKs, His phosphotransfer proteins (HPts), and RRs. Because the SAM method for identifying differentially expressed genes was quite conservative, we chose to take a closer look at the expression levels of several genes encoding His-Asp-signaling components. To summarize the ≥2-fold gene expression differences in His-Asp-signaling components between habituated and nonhabituated callus cultures (Table V), the expression of the cytokinin receptor *CRE1* was highly up-regulated, while that of *AHK3* was moderately down-regulated. Expression of the HPt (positive regulator of cytokinin signaling) *AHP1* was highly down-regulated, while expression of *AHP5* was moderately down-regulated. Two Type-A

Table IV. *Biological process categorization for genes altered in nonhabituated and habituated callus cultures*

Bold text indicates categories in which the percent of up- and down-regulated genes differs by roughly 2-fold or more.

GO Biological Process[a]	Up-Regulated Genes in Nonhabituated Calli[b]	Down-Regulated Genes in Nonhabituated Calli[c]	Up-Regulated Genes in Habituated Calli[d]	Down-Regulated Genes in Habituated Calli[e]
	%	%	%	%
Biological process unknown	12.4	7.2	15	13.3
Cell organization and biogenesis	**1.9**	**0**	2.4	1.5
Developmental processes	2.2	1.4	1.4	1.5
DNA or RNA metabolism	**0.6**	**1.4**	**2.5**	**0.4**
Electron transport or energy pathways	**4.9**	**1.4**	1.6	2.7
Other biological processes	3.9	2.9	4.3	5.7
Other cellular processes	16.6	14.5	16	16.2
Other metabolic processes	16.7	17.4	15.8	16
Other physiological processes	21.5	15.9	17.7	18.7
Protein metabolism	4.3	5.8	**8.4**	**4.2**
Response to abiotic or biotic stimulus	**3.8**	**11.6**	**1.9**	**4**
Response to stress	**1.8**	**14.5**	2	3.1
Signal transduction	1.3	1.4	2	1.8
Transcription	2.7	2.9	4.2	5
Transport	**5.4**	**1.4**	4.5	5.8

[a]Functional categorization is determined by the Gene Ontology Annotation tool on the TAIR Web site. [b]Percentage of the 467 up-regulated genes in nonhabituated calli maintained in the presence of BA (FC + BA), with respect to nonhabituated callus maintained in the absence of BA (FC − BA), falling into each Biological Process category. [c]Percentage of the 23 down-regulated genes in FC + BA, with respect to FC − BA, falling into each Biological Process category. [d]Percentage of the 440 up-regulated genes in habituated calli maintained in the absence of BA (T87 − BA), with respect to nonhabituated callus maintained in the presence of BA (FC + BA), falling into each Biological Process category. [e]Percentage of the 405 down-regulated genes in T87 − BA, with respect to FC + BA, falling into each Biological Process category.

Table V. *Fold-changes in gene expression of cytokinin signaling-related genes*

T87, Calli derived from the T87 habituated cell line; FC, freshly derived nonhabituated calli; BA, the cytokinin benzyl adenine; NA, not available.

Gene Family[a]	Locus Identifier	T87 − BA/ FC + BA[b]	FC + BA/ FC − BA[c]
Cytokinin receptors			
AHK2	At5g35750	0.59	1.24
AHK3	At1g27320	0.28	1.39
CRE1	At2g01830	20.99	1.24
CRE1	At2g01830	18.11	1.27
HKs			
CKI1	At2g47430	0.93	0.80
ATHK1	At2g17820	3.19	1.27
CKI2	At5g10720	0.41	0.75
ETR1	At1g66340	0.70	0.79
ETR2	At3g23150	0.25	2.11
ERS1	At2g40940	0.46	0.99
ERS2	At1g04310	0.66	1.34
EIN4	At3g04580	0.64	1.28
Degenerate HKs			
PHYA	At1g09570	0.28	1.03
PHYB	At2g18790	0.83	0.85
PHYC	At5g35840	1.25	1.21
PHYD	At4g16250	1.22	0.53
PHYE	At4g18130	1.09	1.00
PDK	At3g06483	0.94	1.48
Type-A RRs			
ARR3	At1g59940	0.93	0.78
ARR4	At1g10470	1.87	0.78
ARR5	At3g48100	1.78	1.02
ARR6	At5g62920	1.64	0.57
ARR7	At1g19050	5.12	1.27
ARR8	At2g41310	0.83	1.12
ARR9	At3g57040	1.46	1.75
ARR15	At1g74890	2.85	0.86
ARR16	At2g40670	1.12	0.93
ARR17	At3g56380	0.97	0.96
Type-B RRs			
ARR1-5′	At3g16855	0.70	0.98
ARR1-3′	At3g16857	0.53	1.07
ARR1-3′	At3g16857	0.51	1.07
ARR2	At4g16110	1.38	0.71
ARR10	At4g31920	0.71	1.10
ARR11	At1g67710	0.81	0.80
ARR12	At2g25180	1.44	0.97
ARR13	At2g27070	1.32	0.78
ARR14	At2g01760	1.45	1.69
ARR18	At5g58080	0.96	0.74
ARR19	At1g49190	1.03	0.88
ARR20	At3g62670	1.24	0.60
ARR21	At5g07210	1.17	0.53
ARR22	At3g04820	1.22	0.76
ARR23	At5g62120	1.03	0.71
Pseudo RRs			
APRR1	At5g61380	0.63	1.38
APRR2	At4g18020	0.37	2.24
APRR2	At4g18020	0.33	2.30
APRR2	At4g18020	0.29	2.41
APRR3	At5g60100	0.99	1.05
APRR4	At5g49240	1.23	0.81
APRR5	At5g24470	2.14	1.19
APRR6	At1g68210	1.05	0.98
APRR7	At5g02810	0.81	1.11
APRR8	At4g00760	0.99	1.24
APRR9	At2g46790	1.05	0.27
AHPs			
AHP1	At3g21510	0.08	1.32
AHP2	At3g29350	1.20	1.39
AHP2	At3g29350	1.11	1.43
AHP3	At5g39340	0.66	1.88
AHP4	At3g16360	1.00	0.79
AHP5	At1g03430	0.52	1.73
AHP6	At1g80100	0.99	0.94
Cytokinin-inducible genes			
Cyclin D3	At4g34160	0.55	2.10
KNAT1	At4g08150	0.65	1.37
NR1	At1g77760	0.22	3.31
NR2	At1g37130	0.32	1.23
STM	At1g62360	1.10	1.02
CAB1	At1g29930	0.43	3.48
Cytokinin oxidases			
CKX1	At2g41510	2.43	0.80
CKX2	At2g19500	1.07	0.99
CKX3	At5g56970	10.35	0.70
CKX4	At4g29740	1.06	0.96
CKX4	At4g29740	1.00	0.89
CKX5	At5g21482	0.56	1.51
CKX6	At1g75450	2.00	0.65
CKX7	At3g63440	3.61	1.09
Cytokinin synthases			
IPT1	At1g68460	1.02	0.95
IPT2	At2g27760	0.96	0.68
IPT3	At3g63110	0.43	1.75
IPT4	At4g24650	1.02	0.78
IPT5	At5g19040	0.77	1.15
IPT6	At1g25410	1.13	0.67
IPT7	At3g23630	0.93	0.76
IPT8	At3g19160	1.21	0.69
IPT9	At5g20040	1.14	0.87
IPT9	At5g20040	0.93	0.99
Purine transporters			
AtPUP1	At1g28230	0.80	1.09
AtPUP2	At2g33750	1.06	0.80
AtPUP3	At1g28220	1.15	1.03
AtPUP4	At1g30840	1.41	1.45
AtPUP5	At2g24220	1.14	1.77
AtPUP6	At4g18190	1.15	0.96
AtPUP7/8	At4g18200	1.05	2.71
AtPUP9	At1g18220	1.02	1.07
AtPUP10	At4g18210	1.10	1.22
AtPUP11	At1g44750	0.99	1.13
AtPUP12	At5g41160	1.30	1.27
AtPUP13	At4g08700	0.95	1.39
AtPUP14	At1g19770	0.65	1.77
AtPUP15	At1g75470	1.25	0.72
AtPUP16	At1g09860	1.33	0.78
AtPUP17	At1g57943	1.14	0.96
AtPUP18	At1g57990	1.47	0.66
AtPUP19	At1g47603	NA	NA
AtPUP20	At1g47590	0.94	0.61

[a]The signal intensities for genes represented more than once on the microarray are presented separately. [b]The fold-change for the expression of each gene in habituated calli maintained in the absence of cytokinin is presented relative to the expression of the gene in nonhabituated calli maintained in the presence of cytokinin. [c]The fold-change for the expression of each gene in nonhabituated calli maintained in the presence of cytokinin is presented relative to the expression of the gene in nonhabituated calli maintained in the absence of cytokinin.

RRs, *ARR7* and *ARR15* (negative regulators of cytokinin signaling), were moderately up-regulated. Thus, while the cytokinin receptor *CRE1* was highly up-regulated in the habituated T87 cell line, two HPt proteins that may serve to propagate the cytokinin signal initiated by CRE1 in planta were down-regulated. Furthermore, two RR proteins that may serve to repress the CRE1-initiated His-Asp phosphorelay in planta were up-regulated. These changes may reflect a down-regulation of specific cytokinin-mediated responses in habituated cells. For example, some cytokinin responses were present in the habituated calli (proliferation and greening), whereas other cytokinin responses were lost (shoot induction).

The expression of several documented cytokinin-inducible genes (*CYCD3*, *KNAT1*, *NR1*, *NR2*, *CAB1*) was repressed, rather than up-regulated, in habituated callus tissues (Table V). As expected, expression levels of several of these genes (*CYCD3*, *NR1*, and *CAB1*) were up-regulated by the presence of cytokinin in nonhabituated callus tissues (Table V). No significant changes were seen in the expression levels of genes encoding proteins thought to be involved in cytokinin synthesis (IPTs) in response to cytokinin in either habituated or nonhabituated tissues (Table V). Among the proteins involved in cytokinin degradation (CKXs), *CKX1*, *CKX3*, *CKX6*, and *CKX7* were up-regulated in habituated calli (Table V). Expression levels of the CKXs were not altered in response to cytokinin in nonhabituated calli (Table V).

Other Phytohormone-Related Changes

Several genes involved in hormone biosynthesis were identified by SAM as significantly down-regulated in habituated calli (ethylene synthesis, At2g19590 and At1g12010; GA_3 synthesis, At1g06640, At2g25450, At1g14120, At1g14130, and At2g30840; brassinosteroid synthesis, At2g30490, At1g78490, At2g34490, At1g13080, At2g27000, At2g22330, and At1g12740). The expression of additional genes involved in responses to the plant hormone ethylene was explored further in habituated calli. As shown in Table V, expression of the ethylene receptors *ETR2* and *ERS1* was down-regulated in habituated calli (−4.0-fold and −2.2-fold, respectively). Likewise, expression of several additional ethylene-signaling components was moderately down-regulated in habituated calli (*CTR1*, −1.8-fold; *EIN3*, −1.8-fold; *EIL1*, −1.8-fold; *ERF1*, −1.9-fold; data not shown). Overlaps in the cytokinin- and ethylene-response pathways have been demonstrated. For example, the inhibitory effect of cytokinin on etiolated hypocotyl elongation, as well as root elongation, has been linked to cytokinin-induced ethylene production (Cary et al., 1995). In addition, the Type-B RR *ARR2* has been identified as a transcription factor (Sakai et al., 2000; Lohrmann et al., 2001) that acts as a positive regulator of both cytokinin signaling (Hwang and Sheen, 2001) and ethylene signaling (Hass et al., 2004).

The first committed step in ethylene biosynthesis is catalyzed by 1-aminocyclopropane-1-carboxylic acid synthase (Yang and Hoffman, 1984). While expression of some 1-aminocyclopropane-1-carboxylic acid synthase homologs was up-regulated in habituated calli (*ACS2*, 9.6-fold; *ACS6*, 2.1-fold; *ACS7*, 2.7-fold; *ACS8*, 2.1-fold), expression of others was down-regulated (At1g05010, −2.5-fold; At1g12010, −9.1-fold; At2g19590, −16.7-fold; data not shown). Cytokinin-induced ethylene production, however, occurs primarily through *ACS5* (Vogel et al., 1998), which is unaltered in habituated calli (data not shown). Thus, while our data do not support a role for cytokinin-mediated ethylene production, the down-regulation of several ethylene receptors and signaling components may reflect a negative interaction between ethylene- and cytokinin-signaling pathways in habituated callus cultures.

While a few genes involved in auxin signaling (auxin-responsive proteins At3g15540, At1g15580, and At3g62100) were identified by SAM as up-regulated in habituated calli, others were identified as down-regulated (auxin-induced proteins At1g19840 and At1g72430, auxin-regulated protein At2g33830, and auxin receptor TIR1 homolog At1g12820). The interaction of cytokinin- and auxin-response pathways has been well documented in the organogenesis of plant cell cultures (Skoog and Miller, 1957; Steward, 1970), as well as in the regulation of apical bud dominance (Wickson and Thimann, 1958). Several auxin-related signaling genes, including *TIR1*, *ABP1*, *Aux/IAA*s, *SAUR*s, *ARF*s, and *GH3*s, were analyzed for ≥2-fold changes in gene expression in habituated versus nonhabituated callus cultures.

While the expression of the putative auxin receptor *ABP1* was not altered in habituated calli, the expression of the auxin receptor *TIR1*, as well as several *TIR1* homologs, was down-regulated in habituated calli (*TIR1*, −2.1-fold; At1g12820, −3.8-fold; At3g26810, −2.0-fold; At3g62980, −2.1-fold; At4g03190, −2.6-fold). Out of the 29 Aux/*IAA*s analyzed, three were up-regulated in habituated calli (*IAA5*, 7.1-fold; *IAA19*, 7.9-fold; *IAA30*, 5.5-fold), and six were down-regulated (*IAA2*, −5.9-fold; *IAA9*, −1.9-fold; *IAA13*, −2.1-fold; *IAA16*, −3.3-fold; *IAA27*, −2.4-fold; *IAA28*, −2.1-fold). Out of the 70 SAURs analyzed, three were up-regulated (At4g34750, 2.5-fold; At4g34760, 1.9-fold; At5g53590, 3.3-fold) and five were down-regulated (At1g19840, −16.7-fold; At2g45210, −4.0-fold; At2g46690, −2.5-fold; At4g36110, −2.0-fold; At4g38840, −2.9-fold) in habituated calli. Out of the 23 *ARF*s analyzed, two were down-regulated in habituated calli (*ARF9*, −2.2-fold; *ARF2*, −3.2-fold). Out of the 20 putative *GH3*s analyzed, one was up-regulated (At5g13320, 4.2-fold) and two were down-regulated (At1g28130, −1.9-fold; At4g37390, −2.1-fold) in habituated calli. Hence, no general trends in the alteration of auxin-signaling-related components were seen in habituated calli compared to nonhabituated calli, highlighting the complexity between cytokinin-signaling and auxin-signaling interactions in planta.

Cell Division-Related Changes

Several cell cycle-related proteins (At3g53230, At2g38620, At1g44110, At2g07690, At3g44620), nucleosome components (Histone H2A, At3g20670 and At1g51060; Histone H2B, At3g53650 and At3g09480; Histone H3.2, At1g75600; Histone H4, At3g45930 and At3g46320), and protein and nucleic acid synthesis genes (At2g39590, At3g28500, At3g16780, At1g44900, At3g23890, At4g21710, At2g24050, At3g54490, At4g29090, At3g49000, At2g18720, At2g39820) were identified as up-regulated in habituated calli by the SAM method. Up-regulation of these genes indicates that processes normally up-regulated by cytokinins in nonhabituated tissues, such as DNA replication, protein synthesis, and cell division, are constitutively up-regulated in the habituated T87 cell line.

We chose to take a closer look at the expression levels of several cyclins, cyclin-dependent kinases, and histones in habituated calli with reference to nonhabituated callus (Tables VI and VII). Twelve of 31 cyclins or cyclin homologs (identified using nucleotide BLAST searches against the Arabidopsis genome with identified cyclins) were up-regulated by 2-fold or more in habituated calli, while three were down-regulated by 2-fold or more. Six of 14 cyclin-dependent kinases (CDKs) or CDK homologs (identified using nucleotide BLAST searches against the Arabidopsis genome with identified CDKs) were identified as up-regulated. In terms of nucleosome components (as defined by the Plant Chromatin Database; http://www.chromdb.org/), 7/13 Histone H2A, 8/11 Histone H2B, 9/14 Histone H3, and 6/8 Histone H4 genes were up-regulated in habituated callus cultures. On the other hand, one of three linker Histone H1 genes was down-regulated. While differential expression for many of these histone and cell cycle-related genes was also seen in nonhabituated callus cultures (Tables VI and VII), the transcriptome analysis of these two different tissue types revealed both overlapping and distinct expression changes among gene family members. This result indicates that the accelerated proliferation of habituated T87 cell cultures, with reference to nonhabituated cell cultures, does not simply result from expressing genes normally involved in callus proliferation to a higher degree.

Although habituated callus cultures proliferate more rapidly than nonhabituated callus cultures, many more cell wall growth and modification enzymes were down-regulated in habituated calli (cellulose synthase-like gene, At1g55850; expansins, At2g28950, At1g62980, and At1g12560; extensins, At1g76930 and At1g21310; glycosyl hydrolases, At1g66280, At1g26560, At1g02850, At1g66270, At1g52400, At1g26450, At2g27500, At1g62660, At1g12240, At1g48930, and At2g18360; invertase, At1g47960; pectinesterases, At1g53830, At1g11580, and At1g14890; pectate lyase, At1g67750; UDP-glycosyltransferases, At2g36970, At1g22360, and At1g07240) than up-regulated (glycosyl hydrolases, At3g55430, At2g43620, At2g43570, At4g01700, At3g47540, At3g54420, and At4g01970;

Table VI. *Fold-changes in gene expression of cell division-related genes*

T87, Calli derived from the T87 habituated cell line; FC, freshly derived nonhabituated calli; BA, the cytokinin benzyl adenine.

Gene Family[a]	Locus Identifier	T87 − BA/ FC + BA[b]	FC + BA/ FC − BA[c]
Cyclins			
CYC3B	At5g11300	3.25	1.27
CYC3A	At5g25380	1.11	0.74
CYC2A	At2g17620	1.40	1.01
CYC2B	At4g35620	1.24	1.65
CYC1B	At5g06150	2.68	1.99
CYC1	At4g37490	2.49	1.19
CYCD1	At1g70210	0.52	2.35
CYCD2	At2g22490	0.87	0.62
CYCD3	At4g34160	0.55	2.10
Cyclin homolog	At1g15570	1.55	1.40
Cyclin homolog	At1g80370	1.11	1.16
Cyclin homolog	At1g44110	3.83	1.98
Cyclin homolog	At1g77390	1.13	0.91
Cyclin homolog	At5g43080	1.20	0.90
Cyclin homolog	At1g47210	1.41	1.45
Cyclin homolog	At1g47210	1.47	1.54
Cyclin homolog	At1g47230	1.82	1.27
Cyclin homolog	At1g47220	1.05	0.86
Cyclin homolog	At1g76310	1.74	1.82
Cyclin homolog	At1g20610	1.00	1.21
Cyclin homolog	At1g16330	1.49	1.00
Cyclin homolog	At2g26760	2.58	2.54
Cyclin homolog	At3g11520	2.19	1.28
Cyclin homolog	At1g20590	1.18	1.00
Cyclin homolog	At1g34460	1.75	0.94
Cyclin homolog	At1g14750	1.88	0.76
Cyclin homolog	At5g10440	1.58	0.73
Cyclin homolog	At5g65420	1.37	1.16
Cyclin homolog	At4g37630	1.13	0.79
Cyclin homolog	At3g50070	0.27	0.77
Cyclin homolog	At4g03270	0.92	1.12
Cyclin homolog	At5g67260	0.25	1.59
CDKs			
CDKB2	At1g20930	1.55	1.83
CDKD1	At1g73690	1.83	0.84
CDKB1	At2g38620	3.87	1.22
CDK, subunit 1	At2g27960	0.65	2.46
CDK, subunit 2	At2g27970	3.04	2.41
CDKA1	At3g48750	0.85	1.01
CDKF1	At4g28980	0.91	1.12
CDKF1	At4g28980	0.83	1.17
CDKC2	At5g64960	1.27	0.86
CDKE1	At5g63610	0.90	0.99
CDKC1	At5g10270	1.27	0.85
CDK homolog	At1g18040	1.14	1.11
CDK homolog	At1g66750	1.01	1.02
CDK homolog	At1g76540	2.30	2.81
CDK homolog	At3g54180	3.09	1.80

[a]The signal intensities for genes represented more than once on the microarray are presented separately. [b]The fold-change for the expression of each gene in habituated calli maintained in the absence of cytokinin is presented relative to the expression of the gene in nonhabituated calli maintained in the presence of cytokinin. [c]The fold-change for the expression of each gene in nonhabituated calli maintained in the presence of cytokinin is presented relative to the expression of the gene in nonhabituated calli maintained in the absence of cytokinin.

Table VII. *Fold-changes in expression of histone genes*

T87, Calli derived from the T87 habituated cell line; FC, freshly derived nonhabituated calli; BA, the cytokinin benzyl adenine.

Histone[a]	Locus Identifier	T87 − BA/ FC + BA[b]	FC + BA/ FC − BA[c]
Histone 2A			
HTA1	At5g54640	2.94	1.13
HTA2	At4g27230	2.31	1.83
HTA3	At1g54690	1.85	1.37
HTA4 (H2A.Z)	At4g13570	1.09	0.53
HTA5	At1g08880	0.76	1.42
HTA6	At5g59870	2.39	1.74
HTA7	At5g27670	0.98	1.49
HTA8 (H2A.Z)	At2g38810	1.38	1.29
HTA8 (H2A.Z)	At2g38810	1.28	1.03
HTA8 (H2A.Z)	At2g38810	1.21	0.92
HTA9 (H2A.Z)	At1g52740	0.91	1.02
HTA10	At1g51060	3.17	1.37
HTA11 (H2A.Z)	At3g54560	1.63	2.73
HTA12	At5g02560	0.79	1.56
HTA13	At3G20670	4.56	2.29
Histone 2B			
HTB1	At1g07790	1.59	1.04
HTB2	At5g22880	3.94	4.54
HTB3	At2g28720	1.00	3.84
HTB4	At5g59910	1.14	1.48
HTB5	At2g37470	2.40	1.49
HTB6	At3g53650	5.02	2.00
HTB7	At3g09480	2.93	0.56
HTB8	At1g08170	1.08	0.64
HTB9	At3g45980	1.90	1.79
HTB10	At5g02570	2.15	0.68
HTB11	At3g46030	2.21	1.04
Histone H3			
HTR1	At5g65360	1.91	1.17
HTR2	At1g09200	2.39	1.46
HTR3	At3g27360	2.11	1.91
HTR4 (H3.2)	At4g40030	0.98	1.82
HTR5 (H3.2)	At4g40040	1.03	1.09
HTR6 (H3.2)	At1g13370	3.51	0.86
HTR7	At1g75610	2.62	0.77
HTR8 (H3.2)	At5g10980	1.35	1.23
HTR9	At5g10400	2.49	1.95
HTR10 (H3.2)	At1g19890	1.15	1.02
HTR11	At5g65350	1.20	0.90
HTR12 (CenH3)	At1g01370	1.54	1.31
HTR13	At5g10390	3.19	1.97
HTR14 (H3.2)	At1g75600	5.37	1.02
Histone H4			
HFO1	At3g46320	5.71	2.52
HFO2	At5g59690	2.37	2.21
HFO3	At2g28740	1.78	1.20
HFO4	At1g07820	1.56	1.62
HFO4	At1g07820	1.50	1.31
HFO5	At3g53730	2.34	1.25
HFO6	At5g59970	1.96	1.54
HFO7	At3g45930	5.99	1.91
HFO8	At1g07660	1.28	1.33
Histone H1			
HON1	At1g06760	1.20	1.69
HON2	At2g30620	1.19	1.13
HON3	At2g18050	0.05	2.76
HON3	At2g18050	0.05	2.70

[a]The signal intensities for genes represented more than once on the microarray are presented separately. [b]The fold-change for the expression of each gene in habituated calli maintained in the absence of cytokinin is presented relative to the expression of the gene in nonhabituated calli maintained in the presence of cytokinin. [c]The fold-change for the expression of each gene in nonhabituated calli maintained in the presence of cytokinin is presented relative to the expression of the gene in nonhabituated calli maintained in the absence of cytokinin.

polygalacturonases, At4g23820 and At1g23760; pectinesterases, At3g62060 and At2g01610). This result may reflect a difference in cell wall biochemistry between habituated and nonhabituated cells. Interestingly, a mutation in the *KORRIGAN1/TSD1* gene (an endo-1, 4-β-D-glucanase; Nicol et al., 1998) that results in a decreased cellulose content (Szyjanowicz et al., 2004), a major constituent of plant cell walls, also results in habituation (Zuo et al., 2000; Frank et al., 2002). Furthermore, the *tsd2* mutation leads to vitrified, friable leaf tissue, in addition to habituated callus growth (Frank et al., 2002). Thus, disruption of normal cell wall development and/or adhesion has been linked to habituation. The fold-change of *KORRIGAN1* expression in habituated calli compared to freshly derived calli was −2.2-fold.

Other Interesting Changes

While SAM identified only one transcription factor as up-regulated in habituated calli (WRKY family, At2g03340), 15 were identified as down-regulated (AP2 domain family, At1g78080, At1g22190, and At1g13260; Bhlh related, At2g22770; bZIP family, At1g13600, At2g18160, At1g75390, and At1g77920; heat shock family, At1g46264; myb family, At1g74840, At1g14350, and At1g19000; scarecrow-like, At1g21450 and At1g07530; TCP family, At1g35560; WRKY family, At1g29280). While members of each of these gene families have been identified as cytokinin regulated in other studies, few genes themselves have been identified in these studies. The WRKY-family transcription factor found up-regulated in habituated calli was also found up-regulated by cytokinin in a previous study (Hoth et al., 2003). In contrast, the heat shock transcription factor (Hoth et al., 2003) and Bhlh-related transcription factor (Kiba et al., 2005) found down-regulated in habituated calli were previously identified as cytokinin up-regulated. The fact that we see differential expression of a variety of transcription factors between habituated and nonhabituated callus cultures is not surprising, given the large number of differentially expressed genes between the two tissue types.

Several genes involved in protein degradation, particularly members of the F-box protein family, were identified as either up-regulated (proteasome subunit, At3g22630; F-box protein family, At3g47030, At3g23970, At3g61340, At3g50710, At1g23770, At2g16450, At1g23780, At1g66290, At3g60710, At1g66310, At3g23950, At1g48400, At3g59190, At3g16590, At4g05470, At4g22390, and At3g47130; Kelch repeat-containing F-box protein family, At1g60570 and At4g04670) or down-regulated (F-box protein family, At1g22220, At1g67340, At1g21410, and At2g36090; Kelch repeat-containing F-box protein family, At1g15670, At1g23390, At1g26930, At1g30090, and At1g67480; ubiquitin-conjugating enzyme, At1g63800) in this study. Few reports regarding the involvement of protein degradation in cytokinin signaling have been made to date, and the results are conflicting (Smalle et al., 2002; Yamada et al., 2004). While the

presence of both up- and down-regulation of several genes involved in protein degradation in cytokinin-habituated calli does not simplify the question of whether proteolysis plays an important role in regulating cytokinin signaling, these results do highlight specific genes to investigate further for cytokinin-related phenotypes.

Several calcium-binding proteins were identified as either up-regulated (At2g03450, At3g47480, At4g05520, At4g04695, At4g04720, At3g22910, At3g25600, At4g21820) or down-regulated (At2g18750, At1g25230, At1g76650), indicating a potential role for calcium signaling in cytokinin responses, as has been long expected though direct evidence has been lacking (for review, see Brault and Maldiney, 1999; Faure and Howell, 1999).

Evidence for Epigenetic Modifications in Habituated Callus Cultures

Notably, the expression of several transposon-related elements was up-regulated in habituated T87 callus cultures (At3g43563, At3g43862, At3g42253, At4g08680, At1g78095, At2g30640, At3g42806, At1g49090, At2g14230; Supplemental Table IV), as identified by SAM. A closer look revealed up-regulation of 2-fold or more for 37/485 transposon-related elements represented on the microarray (Table VIII). Activation of transposons during the process of plant cell culture has been documented previously (for review see Kaeppler et al., 2000) and has also been shown to correlate with changes in DNA methylation patterns (for review see Bender, 2004).

DNA methylation states are important for silencing or activation of gene expression and also play a role chromatin structure. In general, a silenced transcriptional state is correlated with higher levels of cytosine methylation, while an active transcriptional state is correlated with lower levels of cytosine methylation. These alterations in DNA methylation are maintained during the culture process, passed to plants regenerated from these callus cultures, and even passed to the progeny of plants regenerated from cultured cells (Kaeppler et al., 2000). Histone modifications, including methylation and acetylation of particular Lys residues, are also involved in chromatin structure and gene silencing (for review, see Loidl, 2003; Bender, 2004). In transcriptionally silent heterochromatin, for example, DNA methylation is often accompanied by Histone 3 methylation of Lys-9 (H3K9met), as well as Histone 4 hypoacetylation. In transcriptionally active euchromatin, on the other hand, acetylation of Histone 4 is often accompanied by demethylation of H3K9 and methylation of H3K4 (Nishioka et al., 2002; Peters et al., 2002; Hashimshony et al., 2003). The up-regulation of several transposon-related elements and chromatin remodeling factors (At4g31900, At1g80740, At1g69770; Supplemental Table IV) in habituated callus cultures warranted a scan of the expression levels of DNA and histone modification enzymes in T87 habituated calli.

Arabidopsis utilizes three classes of DNA methyltransferases that transfer a methyl group from *S*-adenosylmethionine to the C5 position of cytosine residues: methyltransferases (METs), chromomethylases (CMTs), and domains rearranged methylases (DRMs). The METs maintain CpG methylation (Cao and Jacobsen, 2002b; Tariq et al., 2003), CMTs maintain non-CpG methylation (Cao and Jacobsen, 2002b), and DRMs initiate de novo cytosine methylation at CpG, CpNpG, and asymmetric sites (Cao and Jacobsen, 2002a). DRMs also play a role in maintenance of methylation at CpNpG and asymmetric sites (Cao and Jacobsen, 2002b). The Arabidopsis genome encodes four putative METs. Of these, only *MET1* was altered in habituated calli (3.5-fold up-regulation; Table IX). The Arabidopsis genome also encodes four putative CMTs. Of these, both *CMT1* and *CMT3* were altered in habituated calli (5.5-fold and 3.2-fold induction, respectively; Table IX). Three putative DRMs are encoded by the Arabidopsis genome. Of these, expression of *DRM1* was altered in habituated calli (2.1-fold up-regulation; Table IX). The Arabidopsis genome encodes four putative DNA glycosylases, which act to demethylate cytosine residues (for review, see Chan et al., 2005). One of these genes, *DNG1*, was down-regulated 2.0-fold in habituated calli.

Of the 39 putative histone methyltransferases encoded by the Arabidopsis genome, five were up-regulated in habituated calli (1.8–2.1-fold induction) and one was down-regulated (2-fold; Table IX). Methylation of DNA and proteins depends on the methyl donor *S*-adenosylmethionine. Production of *S*-adenosylmethionine occurs through three key biosynthetic steps catalyzed by cystathionine γ-synthetase, cystathionine β-lyase, and Met synthase, respectively (for review see Hesse and Hoefgen, 2003). Expression levels of cystathionine γ-synthetase, cystathionine β-lyase, and Met synthase homologs were not altered in habituated calli (data not shown). Thus, the up-regulation of several DNA and histone methyltransferases was not simply the result of an up-regulation in *S*-adenosylmethionine production. Expression of 3/14 histone acetyltransferase family members in Arabidopsis was up-regulated in habituated calli (1.6–3.8-fold). Likewise, 6/23 histone deacetlyases were up-regulated (1.7–3.4-fold). Several putative chromatin remodeling factors (12/49) were also up-regulated in habituated calli (1.7–7.8-fold; Table IX).

Surprisingly, expression of the *FWA* gene (At4g25530) was up-regulated approximately 87-fold in habituated calli (Supplemental Table IV). FWA is a homeodomain-containing transcription factor that is important for the transition to flowering, as well as for floral meristem identity (Soppe et al., 2000). *FWA* expression is normally confined to the central cell of the female gametophyte and the endosperm (Kinoshita et al., 2003). Hypomethylation of the 5′ region of *FWA* leads to ectopic expression and causes a delay in flowering (Soppe et al., 2000). This is an interesting case in which the methylated (silenced) state of *FWA* is the

Table VIII. *Fold-changes of transposon-related element expression*

T87, Calli derived from the T87 habituated cell line; FC, freshly derived nonhabituated calli; BA, the cytokinin benzyl adenine.

NimbleGen Probe ID	Gene Name	Common Name	T87 − BA/ FC + BA[a,b]	FC + BA/ FC − BA[b,c]
ATHA0004S00021937	At4g08680	MuDR-A transposon protein related	177.69	1.08
ATHA0004S00004017	At1g78095	Mutator-related transposase	106.42	1.10
ATHA0004S00025253	At5g27345	Mutator-related element with long TIRs	101.84	1.54
ATHA0004S00026213	At5g35054	Transposon protein related	23.01	1.19
ATHA0004S00014187	At3g21040	Copia-like retrotransposon family	12.23	0.70
ATHA0004S00026952	At5g35792	Mutator-related transposase related	11.85	0.84
ATHA0004S00013694	At3g43563	Athila retroelement ORF2 related	11.82	0.92
ATHA0004S00013783	At3g43862	Athila retroelement ORF2 related	10.25	0.66
ATHA0004S00003247	At1g49090	Plant transposase (Ptta/En/Spm) family	8.98	1.15
ATHA0004S00014185	At3g21030	Copia-like retrotransposon family	7.60	0.71
ATHA0004S00025144	At5g33395	Plant transposase (Ptta/En/Spm) family	5.29	0.84
ATHA0004S00014177	At3g20990	Copia-like retrotransposon family	4.74	0.90
ATHA0004S00002709	At1g32590	Copia-type polyprotein related	4.47	0.94
ATHA0004S00008664	At2g14230	Plant transposase (Ptta/En/Spm) family	4.40	0.95
ATHA0004S00016405	At3g42253	Athila retroelement ORF2 related	4.12	0.86
ATHA0004S00014179	At3g21010	Copia-like retrotransposon family	4.17	0.70
ATHA0004S00023293	At5g32475	Athila retroelement ORF2 related	3.60	0.64
ATHA0004S00023218	At5g32306	Athila retroelement ORF2 related	3.27	0.74
ATHA0004S00017990	At3g42806	Mutator-related transposase	3.06	0.87
ATHA0004S00015448	At3g33067	Athila retroelement ORF2 related	3.00	0.98
ATHA0004S00008019	At2g13310	Plant transposase (Ptta/En/Spm) family	2.97	0.98
ATHA0004S00010957	At2g04770	Plant transposase (Ptta/En/Spm) family	2.89	0.87
ATHA0004S00008228	At2g30640	Mutator-related transposase	2.88	0.81
ATHA0004S00008982	At2g09187	Athila ORF1 (Arabidopsis) related	2.82	1.25
ATHA0004S00027830	At5g36655	Plant transposase (Ptta/En/Spm) family	2.77	0.80
ATHA0004S00021430	At4g04590	Transposon protein related	2.70	0.93
ATHA0004S00013674	At3g43523	Ac-related transposase	2.62	0.96
ATHA0004S00013670	At3g43510	Copia-like retrotransposon family	2.62	0.89
ATHA0004S00006548	At1g43840	Plant transposase (Ptta/En/Spm) family	2.52	0.73
ATHA0004S00017917	At3g42716	Athila retroelement ORF2 related	2.45	0.71
ATHA0004S00011669	At2g05650	En/Spm-related transposon protein	2.39	0.65
ATHA0004S00011836	At2g23500	Mutator-related transposase	2.24	0.73
ATHA0004S00008009	At2g13260	Athila retroelement ORF1 protein related	2.22	0.84
ATHA0004S00011656	At2g12240	Plant transposase (Ptta/En/Spm) family	2.12	1.16
ATHA0004S00017980	At3g42792	Mutator-related transposase	2.08	0.78
ATHA0004S00001359	At1g36460	Plant transposase (Ptta/En/Spm) family	2.03	0.78
ATHA0004S00009369	At2g14950	Ac-related transposase	2.02	0.94

[a]The fold-change for the expression of each gene in habituated calli maintained in the absence of cytokinin is presented relative to the expression of the gene in nonhabituated calli maintained in the presence of cytokinin. [b]The transposon-related elements whose expression differed by a ≥2-fold change between habituated and nonhabituated calli are presented. [c]The fold-change for the expression of each gene in nonhabituated calli maintained in the presence of cytokinin is presented relative to the expression of the gene in nonhabituated calli maintained in the absence of cytokinin.

default state in all tissues, while endosperm-specific expression of the gene requires DNA demethylation. The regulation of FWA expression is accomplished, at least in part, by the DEMETER DNA glycosylase (Kinoshita et al., 2003). The *DEMETER* transcript was not altered in T87 calli with respect to nonhabituated calli (Table IX). Other genes for which differential expression has been detected based on promoter or gene methylation state (*SUPERMAN*, AT3G23130; *PAI1*, AT1G07780; *PAI2*, AT5G05590; *PAI3*, AT1G29410) were not differentially expressed in habituated calli (data not shown). This result indicates that the alterations in gene expression seen between habituated and nonhabituated callus cultures do not result simply from global hypomethylation of DNA.

Verification of Microarray Results by RT-PCR

Several genes whose expression was altered to varying degrees in habituated calli were chosen for verification of the microarray results. The results of reverse transcription (RT)-PCRs performed on serial dilutions prepared from habituated and freshly derived callus tissues were in agreement with the alterations in gene expression detected by the microarray analysis. For these experiments, cDNA aliquots were taken from the same samples used for hybridization to the microarray. As can be seen in Supplemental Figure 1, this agreement was seen for the direction of change, and was also generally seen for the magnitude of change, in gene expression. For example, the microarray

Table IX. *Fold-changes in expression of epigenetic-related genes*

T87, Calli derived from the T87 habituated cell line; FC, freshly derived nonhabituated calli; BA, the cytokinin benzyl adenine.

Gene Family[a]	Locus Identifier	T87 − BA/ FC + BA[b]	FC + BA/ FC − BA[c]
Histone deacetylases			
HDT1	At3g44750	2.82	0.71
HDT2	At5g22650	3.43	0.66
HDT2	At5g22650	3.18	0.74
HDT3	At5g03740	1.31	0.78
HDT4	At2g27840	1.14	0.67
HDT4	At2g27840	1.02	0.82
HDA2	At5g26040	0.81	0.88
HDA5	At5g61060	0.79	1.26
HDA6	At5g63110	1.19	0.89
HDA7	At5g35600	1.05	0.79
HDA8	At1g08460	0.69	1.04
HDA9	At3g44680	2.16	0.94
HDA10	At3g44660	1.74	1.10
HDA14	At4g33470	0.86	1.40
HDA15	At3g18520	0.84	1.01
HDA15	At3g18520	0.91	0.88
HDA17	At3g44490	2.25	0.95
HDA18	At5g61070	2.79	0.70
HDA19	At4g38130	1.51	0.76
SRT1	At5g55760	0.79	0.90
SRT2	At5g09230	1.22	0.86
SRT2	At5g09230	1.08	1.21
SRT2	At5g09230	1.11	1.20
SRT2	At5g09230	1.02	1.11
SNT1	At1g24190	0.78	0.80
SNT2	At1g70060	1.04	0.88
SNT3	At3g01320	1.12	0.96
SNT4	At5g15020	0.65	1.14
HCP1	At2g45640	0.97	0.82
Histone acetyltransferases			
HAC1	At1g79000	0.77	1.16
HAC2	At1g67220	3.84	0.57
HAC4	At1g55970	0.91	0.97
HAC5	At3g12980	1.40	0.87
HAC12	At1g16710	0.72	0.98
HAG1	At3g54610	0.94	1.22
HAG2	At5g56740	1.18	0.91
HAG3	At5g50320	0.95	0.85
HAM1	At5g64610	1.58	1.21
HAM2	At5g09740	1.09	0.90
HAF1	At1g32750	1.67	0.86
HAF2	At3g19040	1.18	0.67
HXA1	At4g16420	0.84	0.98
HXA2	At3g07740	0.98	0.95
Histone methyltransferases			
SDG1/SET1	At2g23380	1.03	0.95
SDG2/SET2/ATXR3	At4g15180	1.26	0.88
SDG3/SET3/SUVH2	At2g33290	1.12	1.13
SDG4/SET4/ASHR3	At4g30860	1.25	1.08
SDG5/SET5/FIS1/MEA	At1g02580	0.96	0.74
SDG6/SET6/SUVR5	At2g23750	1.14	0.93
SDG7/SET7/ASHH3	At2g44150	0.50	0.97
SDG8/SET8/ASHH2	At1g77300	1.18	0.78
SDG9/SET9/SUVH5	At2g35160	0.76	0.85
SDG10/SET10/EZA1	At4g02020	1.88	1.08
SDG11/SET11/SUVH10	At2g05900	1.06	1.10
SDG13/SET13/SUVR1	At1g04050	1.34	1.09
SDG14/SET14/ATX3	At3g61740	1.47	0.82
SDG15/SET15/ATXR5	At5g09790	1.17	0.91
SDG16/SET16/ATX4	At4g27910	1.28	1.00
SDG17/SET17/SUVH7	At1g17770	0.98	0.86
SDG18/SET18/SUVR2	At5g43990	2.11	1.11
SDG19/SET19/SUVH3	At1g73100	0.99	1.21
SDG20/SET20/SUVR3	At3g03750	1.29	0.98
SDG21/SET21/SUVH8	At2g24740	1.09	0.90
SDG22/SET22/SUVH9	At4g13460	1.32	1.04
SDG23/SET23/SUVH6	At2g22740	1.37	0.94
SDG24/SET24/ASHH4	At3g59960	0.97	0.72
SDG25/SET25/ATXR7	At5g42400	0.94	1.03
SDG26/SET26/ASHH1	At1g76710	0.70	1.19
SDG27/SET27/TRX1	At2g31650	1.84	0.81
SDG29/SET29/ATX5	At5g53430	1.24	1.04
SDG30/SET30/ATX2	At1g05830	0.94	1.45
SDG31/SET31/SUVR4	At3g04380	1.46	0.82
SDG32/SET32/SUVH1	At5g04940	1.28	0.99
SDG32/SET32/SUVH1	At5g04940	1.40	0.86
SDG33/SET33/ SUVH4/KYP	At5g13960	1.79	1.30
SDG34/SET34/ATXR6	At5g24330	2.08	0.91
SDG35/SET35/ATXR1	At1g26760	1.10	1.02
SDG36/SET36/ATXR2	At3g21820	1.05	0.86
SDG37/SET37/ASHR1	At2g17900	1.45	0.89
SDG38/SET38/ATXR4	At5g06620	1.16	1.18
SDG39/SET39/ASHR2	At2g19640	1.72	0.61
SDG39/SET39/ASHR2	At2g19640	1.35	0.65
SDG40	At5g17240	1.30	0.98
SDG41	At1g43245	0.64	1.01
Chromatin remodeling			
CHB1	At2g47620	0.95	1.10
CHB2	At2g33610	0.59	1.74
CHB3	At4g34430	1.06	0.96
CHB3	At4g34430	1.09	1.01
CHB3	At4g34430	1.12	1.03
CHB4	At1g21700	0.63	0.95
CHC1	At5g14170	1.03	1.08
CHC2	At3g01890	3.12	0.85
CHE1	At3g17590	1.10	1.32
CHR1/DDM1	At5g66750	2.31	0.81
CHR2	At2g46020	0.78	1.26
CHR3/SYD	At2g28290	0.94	0.79
CHR3/SYD	At2g28290	0.81	0.77
CHR4	At5g44800	1.14	1.05
CHR5	At2g13370	0.96	0.90
CHR6/PKL	At2g25170	1.07	0.94
CHR7	At4g31900	7.82	0.76
CHR8	At2g18760	0.99	0.71
CHR9	At1g03750	0.78	1.01
CHR10	At2g44980	0.83	1.33
CHR11	At3g06400	0.60	1.12
CHR12	At3g06010	0.80	0.87
CHR13	At3g12810	1.12	0.98
CHR14	At5g07810	1.77	0.79
CHR15/MOM	At1g08060	1.08	0.85
CHR15/MOM	At1g08060	1.23	0.84
CHR16	At3g54280	1.20	0.95
CHR17	At5g18620	1.18	0.86
CHR17	At5g18620	1.13	0.95
CHR18	At1g48310	1.20	0.95
CHR19	At2g02090	0.78	0.93
CHR20	At1g08600	1.05	0.79
CHR21	At3g57300	0.77	1.10
CHR22	At5g05130	1.49	1.18
CHR23	At5g19310	1.45	0.69
CHR24	At5g63950	2.08	1.08
CHR25	At3g19210	1.42	0.75
CHR26	At3g16600	1.34	0.73
CHR27	At3g20010	1.52	0.75
CHR28	At1g50410	1.16	1.20
CHR29	At5g22750	1.92	0.93

(*Table continues on following page.*)

Table IX. (*Continued from previous page.*)

Gene Family[a]	Locus Identifier	T87 − BA/ FC + BA[b]	FC + BA/ FC − BA[c]
CHR30	At1g11100	1.24	0.53
CHR31	At1g05490	2.00	0.89
CHR32	At5g43530	1.22	0.78
CHR33	At1g61140	1.05	0.93
CHR34	At2g21450	1.85	0.84
CHR35/DRD1	At2g16390	1.28	1.15
CHR36	At2g40770	0.78	1.02
CHR37	At1g05120	1.00	1.11
CHR38	At3g42670	2.44	1.16
CHR39	At3g54460	0.94	1.36
CHR40	At3g24340	2.62	0.85
CHR41	At1g02670	2.74	0.87
CHR42	At5g20420	1.10	0.89
DNA methyltransferases			
MET1/DDM2/DMT1	At5g49160	3.51	1.33
MET2/DMT2	At4g14140	0.95	0.92
MET3/DMT3	At4g13610	1.24	0.97
METIIb/DMT8	At4g08990	1.22	0.94
CMT1/DMT4	At1g80740	5.48	1.03
CMT2/DMT5	At4g19020	0.93	1.52
CMT3/DMT3	At1g69770	3.16	1.48
DRM1/DMT9	At5g15380	2.05	0.73
DRM2/DMT7	At5g14620	0.77	1.05
DRM3/DMT10	At3g17310	1.44	1.33
DRM3/DMT10	At3g17310	1.34	1.34
DMT11/DNMT2	At5g25480	1.09	1.07
DNA glycosylases			
DNG1	At2g36490	0.50	1.43
DNG2	At3g10010	1.28	0.83
DNG3/DEMETER	AT5G04560	0.99	1.05
DNG4	At4g34060	1.01	0.94
Dicer-like proteins			
DCL1	At1g01040	0.71	0.93
DCL2	At3g03300	0.95	1.03
DCL3	At3g43920	2.48	1.33
DCL4	At5g20320	0.67	1.03
Histone demethylases			
HDMA1	At3g10390	0.99	0.83
HDMA3	At1g62830	0.80	1.00
HDMA2	At3g13682	1.60	0.75
HDMA4	At4g16310	1.30	0.81

[a]The signal intensities for genes represented more than once on the microarray are presented separately. [b]The fold-change for the expression of each gene in habituated calli maintained in the absence of cytokinin is presented relative to the expression of the gene in nonhabituated calli maintained in the presence of cytokinin. [c]The fold-change for the expression of each gene in nonhabituated calli maintained in the presence of cytokinin is presented relative to the expression of the gene in nonhabituated calli maintained in the absence of cytokinin.

analysis revealed that the expression of *CRE1* was up-regulated by 19.6-fold in habituated calli (Table V). By RT-PCR analysis, the *CRE1* transcript could barely be amplified from a 100-fold dilution of cDNA prepared from freshly derived calli, while this transcript could still be amplified from a 10,000-fold dilution of cDNA prepared from habituated calli (Supplemental Fig. 1). In contrast, microarray analysis revealed a reduction in *AHP1* expression by 12.5-fold (Table V). By RT-PCR analysis, the *AHP1* transcript could be amplified from a 10,000-fold dilution of cDNA prepared from freshly derived calli, while this transcript could be amplified to approximately the same degree from a 100-fold dilution of cDNA prepared from habituated calli (Supplemental Fig. 1). In addition, the microarray analysis revealed an up-regulation of *FWA* expression by about 86-fold, of *AtHK1* expression by 3.2-fold, and of *ARR5* expression by 1.8-fold (Table V; Supplemental Table IV). By RT-PCR analysis, the *FWA* transcript was undetectable even in undiluted cDNA prepared from freshly derived calli, while this transcript could be amplified from a 10,000-fold dilution of cDNA prepared from habituated calli. The *AtHK1* transcript, on the other hand, could be amplified from a 100-fold dilution of cDNA prepared from freshly derived calli and from a 1,000-fold dilution of cDNA prepared from habituated calli. The *ARR5* transcript could be amplified from a 10,000-fold dilution of cDNA prepared from both freshly derived and habituated calli, but the intensity of the amplified transcript was slightly less in freshly derived calli (Supplemental Fig. 1).

Several of these genes were also chosen for quantitative RT-PCR analysis (qPCR) on cDNA prepared both from RNA isolated from the same samples used for hybridization to the microarray and from RNA isolated from separate habituated and nonhabituated callus tissues handled the same way as those used for microarray hybridization (Table X). Fold-changes in transcript abundance between habituated and nonhabituated callus cultures were calculated based on average count numbers normalized to the abundance of an Actin2 control transcript (for variations in control gene expression, see Table XI). In all cases, the direction of differential expression (i.e. up- or down-regulation) calculated for each transcript based on microarray analysis or qPCR was the same, and in most cases (*CRE1*, *AHK2*, *AHK3*, *AtHK1*, *AHP1*) the fold-changes calculated by both methods were numerically very close to one another (Table X). While there were a small number of discrepancies between the two methods (namely, for the *TMK3* and *FWA* transcripts), whether one favors the qPCR or the microarray data does not change our conclusions. Thus, based on these two independent PCR-based validation methods, which were performed with different gene-specific primer pairs, we felt confident that the fold-changes calculated by microarray analysis accurately reflected genome-wide transcriptome-based changes between habituated and nonhabituated callus cultures.

CRE1 Protein Quantification

The completion of the Arabidopsis genome sequence, coupled with the development of DNA microarray technologies, has made it possible to analyze genome-wide mRNA expression patterns (i.e. the transcriptome) within whole plants (Rashotte et al., 2003; Bergmann et al., 2004), specific plant tissues (Che et al., 2002; Himanen et al., 2004), and even particular cells within a tissue type (Birnbaum et al., 2003). It is the changes in protein expression rather than the changes in mRNA expression, however, that truly

Table X. *Comparison of chip data to qPCR data*

Gene	T87 − BA/ FC + BA Average Fold-Change, Microarray[a]	T87 − BA/ FC + BA Average Fold-Change, qPCR[b]	Hab1 − BA/ FC + BA Average Fold-Change, qPCR[b]
CRE1	20.99, 18.11[c]	21.94	−16.25
AHK2	−1.69	−4.00	−3.69
AHK3	−3.62	−2.94	−1.40
FWA	86.73	5,151.70	42.39
TMK3	6.24	20.30	2.53
AtHK1	3.19	3.25	1.28
AHP1	−12.07	−9.05	8.42

[a]The fold-change for the expression of each gene in habituated calli maintained in the absence of cytokinin is presented relative to the expression of the gene in nonhabituated calli maintained in the presence of cytokinin, as determined by microarray results. [b]qPCR was performed on three biological replicates of RNA from each sample type (T87 − BA, FC + BA, and Hab1), with two to four technical replicates per sample, per gene. Numbers listed are averages of every qPCR reaction performed, normalized to an Actin control reaction. [c]The microarray has CRE1 represented twice.

reflect how a plant's growth and development are regulated in response to various internal and external cues. Recent studies comparing transcriptome and proteome profiles in yeast (*Saccharomyces cerevisiae*; Gygi et al., 1999) and human synovial tissue (Lorenz et al., 2003) have indicated that in many instances transcript levels are not good predictors of the corresponding protein levels. For this reason, we sought to quantify the level of CRE1 protein expression in habituated callus tissue.

A method has been developed enabling the absolute quantification (AQUA) of a protein from a complex mixture, by directly comparing the relative levels of a native tryptic peptide from the protein of interest with that of a known quantity of synthetic, isotopically labeled peptide standard (Gerber et al., 2003). In this case, the protein of interest was CRE1, the complex mixture was a total membrane protein fraction isolated from habituated or nonhabituated callus tissues, and the synthetic peptide was SS[L*]PENPTVEER, where L* refers to an isotopically labeled Leu residue ([$^{13}C_6$,^{15}N]Leu). This synthetic peptide corresponds to a unique tryptic peptide within the putative extracellular, ligand-binding domain of CRE1.

A schematic of the protocol used for CRE1 quantitation by the AQUA method is outlined in Figure 3. Total membrane protein fractions were independently isolated from habituated and nonhabituated callus tissues, trypsinized, spiked with the isotopically labeled CRE1 internal standard peptide, and introduced into a triple-quadrupole mass spectrometer by electrospray ionization after online reversed-phase HPLC separation. The y_9 (2+) fragment ion from the parent peptide (SSLPENPTVEER) was chosen for selected reaction monitoring, on the basis of its strong signal. Representative extracted ion chromatograms of the CRE1-selected fragment ion, from habituated (blue) and nonhabituated (magenta) callus cultures, are presented in Figure 4. Based on the ratio of the area under the curve for the native and synthetic CRE1 fragment ions, as well as the known initial quantity of synthetic CRE1 peptide added to the callus protein mixture, the quantity of CRE1 in 45 μg total membrane protein was determined. For T87 calli, the quantity of CRE1 protein was 0.477 ± 0.041 pmol (n = 5). For freshly derived calli, the quantity of CRE1 protein was 0.0247 ± 0.0026 pmol (n = 5). These differences correspond to an approximately 19-fold increase in CRE1 protein levels of habituated calli with respect to nonhabituated calli (0.477 pmol/0.0247 pmol). Thus, the approximately 19-fold induction of *CRE1* mRNA expression in habituated callus tissue corresponds to an approximately 19-fold induction of CRE1 protein expression.

Sequencing Analysis of Select Promoter and Gene Sequences within Habituated Calli

To explore the hypothesis that a mutation is responsible for overexpression of *CRE1* in habituated tissues, the promoter and coding regions for three genes, *CRE1*, *TMK3*, and *AHP1*, were selected for sequencing from genomic DNA prepared from habituated callus cultures. *TMK3* was chosen for analysis because it is the gene immediately downstream of *CRE1* and is also up-regulated in habituated calli (6.2-fold). *AHP1* was chosen for analysis because it is a representative down-regulated gene in habituated calli (Table V). Comparison of the *AHP1* and *TMK3* promoter and coding region sequences from T87 cells to that generated by the Arabidopsis Genome Initiative (2000) revealed no base changes specific to habituated calli. Sequence analysis of the *CRE1* coding region revealed one putative base change specific to habituated T87 callus cultures: T181C within exon 5. This nucleotide change corresponds to an amino acid change, F453L, within the HK domain of the CRE1 protein sequence (Fig. 5).

Table XI. *Variation of Actin control gene expression*

qPCR was performed on three biological replicates of RNA from each sample type (T87 − BA, FC + BA, and Hab1), with two to four technical replicates per sample, per gene. Ave. count # refers to the average threshold cycle number for amplification of the Actin2 transcript, determined by the iCYCLER at an annealing temperature of 59°C or 60.5°C.

Actin2	Anneal Temperature 59°C		
	T87 − BA	FC + BA	Hab1
Ave. count #	19.12	19.78	19.51
SD	1.19	1.74	1.36
	Anneal Temperature 60.5°C		
	T87 − BA	FC + BA	Hab1
Ave. count #	19.54	19.28	20.75
SD	1.52	1.74	1.64

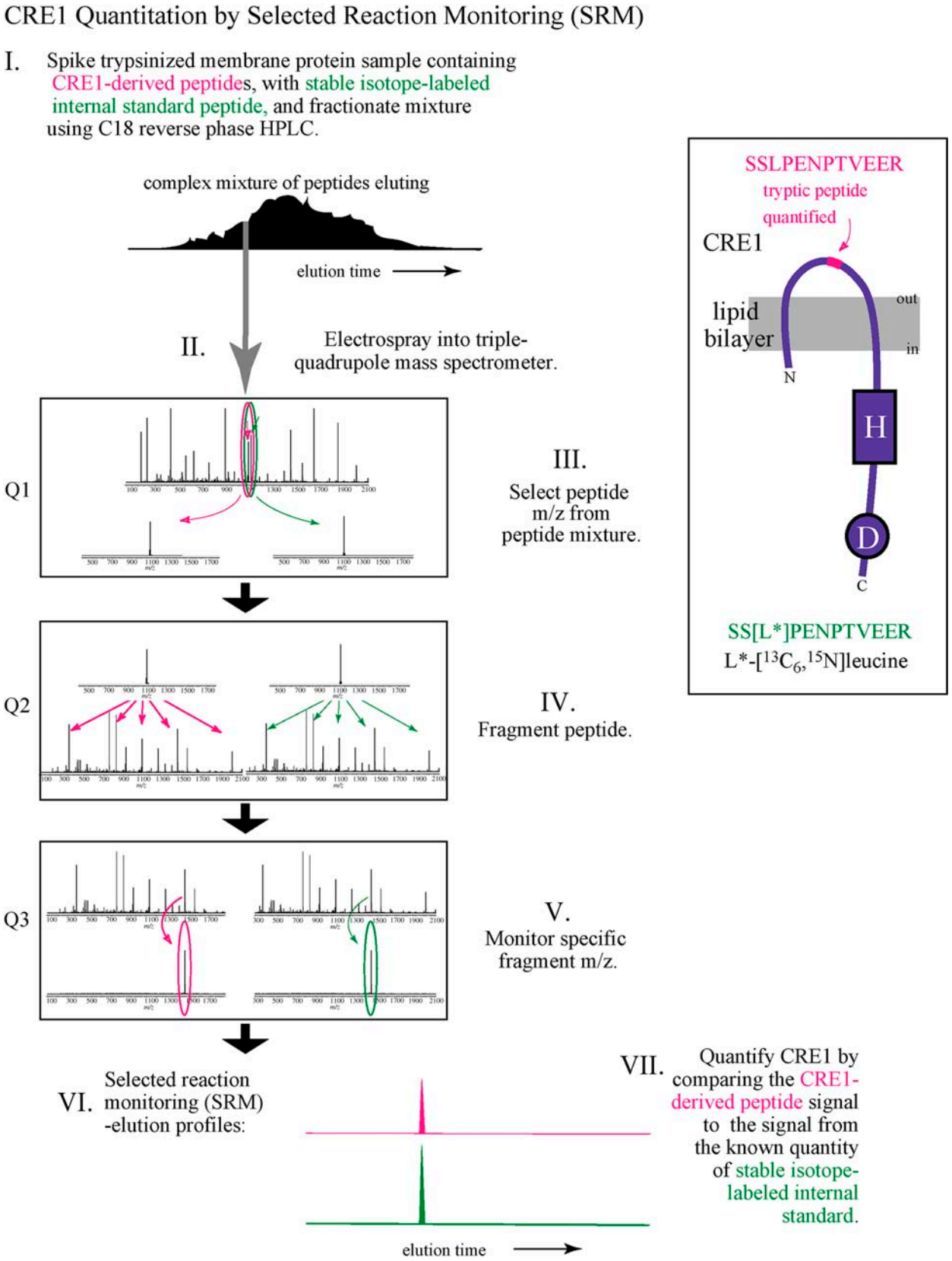

Figure 3. Schematic of the CRE1 protein quantification protocol.

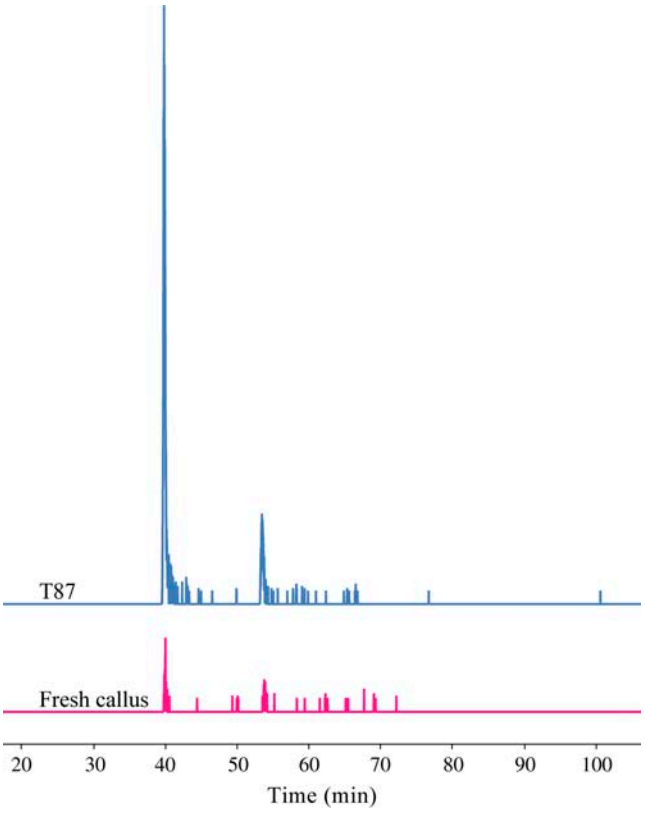

Figure 4. Selected reaction monitoring reveals a 19-fold increase in CRE1 expression in habituated (T87) calli relative to nonhabituated (Fresh callus) calli. Representative extracted ion chromatograms of the selected fragment ion, from the CRE1-derived peptide, are shown. Blue, Habituated callus culture; magenta, nonhabituated callus culture.

Isolation of New Habituated Cell Lines

We generated several independent lines of habituated callus cultures by continued passage of a freshly derived, nonhabituated callus culture. A comparison of the cytokinin responses of root-derived callus cultures (ecotype Columbia [Col]) after four successive passages onto fresh media containing both auxin and cytokinin, followed by seven successive passages of the same callus cultures onto fresh media either containing or lacking cytokinin, is shown in Figure 6. The Hab1 line is a good candidate for a habituated cell line because it proliferated and turned green in the absence of cytokinin but was inhibited by the presence of cytokinin, similar to the T87 cell line. The Hab3 line appeared to proliferate more rapidly in the presence of cytokinin, but still grew and had a greening response in the absence of cytokinin. The Hab4 line seemed to proliferate and turn green well in the absence of cytokinin, but was not particularly inhibited by the presence of cytokinin. Thus, while we see quite a bit of phenotypic variation, clearly we have been able to isolate callus lines that can be maintained in the absence of cytokinin and are thus cytokinin habituated. As Hab1 appeared to be the most promising cytokinin-habituated callus culture line, we used qPCR to analyze the expression levels of select genes, including *CRE1*, relative to nonhabituated callus cultures (Table X). While we did not see up-regulation of the cytokinin receptor *CRE1* in the Hab1 line, we did see up-regulation of the cytokinin-signaling component *AHP1* (8.4-fold), lending support to the idea that habituation occurs via aberrant expression of cytokinin-signaling components. Similar to the T87 cell line (Supplemental Table IV), we also saw up-regulation of *FWA* (42.4-fold) in the Hab1 line. Because it is well documented that up-regulation of *FWA* expression occurs via hypomethylation within the promoter and 5′ region of the gene (Soppe et al., 2000; Cao and Jacobsen, 2002a; Kinoshita et al., 2003), this result lends support to the hypothesis that epigenetic changes have occurred in the Hab1 habituated callus line.

DISCUSSION

Cytokinins are a family of N^6-substituted adenine derivatives whose stimulation of cell division and organogenesis in plants has been studied since the discovery of the first cytokinin, kinetin, in 1956 (Miller et al., 1956). The isolation of triple mutants in the Arabidopsis cytokinin-receptor family (*AHK2*, *AHK3*, and *CRE1*) has confirmed that cytokinins are essential for appropriate plant growth and development, as these plants are infertile and have severely stunted growth (Higuchi et al., 2004). Even so, acquisition of a cytokinin-independent growth habit, also called cytokinin habituation, has been documented in plant cell culture for several different species (Boeken et al., 1974; Meins and Foster, 1986; Axelos et al., 1992). We have used whole-genome microarray analysis of Arabidopsis callus cultures as a new approach toward exploring an old question: What is the mechanism of cytokinin habituation? We used SAM (Tusher et al., 2001) to identify more than 800 significantly differentially expressed genes between habituated and nonhabituated callus cultures. Altered expression of the cytokinin receptor *CRE1* in habituated callus cultures prompted a closer look at the expression of genes encoding cytokinin signal transduction components.

A growing body of evidence indicates that transduction of the cytokinin signal in Arabidopsis is carried out by means of a His-Asp phosphorelay, involving three key proteins: HKs, HPts, and RRs. Because habituated callus cultures no longer respond normally to cytokinin, we examined the expression of genes involved in cytokinin responses in habituated calli with respect to nonhabituated freshly derived calli (Table V). Strikingly, the expression level of the cytokinin receptor *CRE1* was up-regulated 19-fold in habituated calli. Changes in the level of *CRE1* expression have been reported, in response to increasing durations of cytokinin exposure, in Arabidopsis seedlings (approximately 4-fold induction after 24 h of cytokinin exposure [Rashotte et al., 2003]) as well as callus tissue

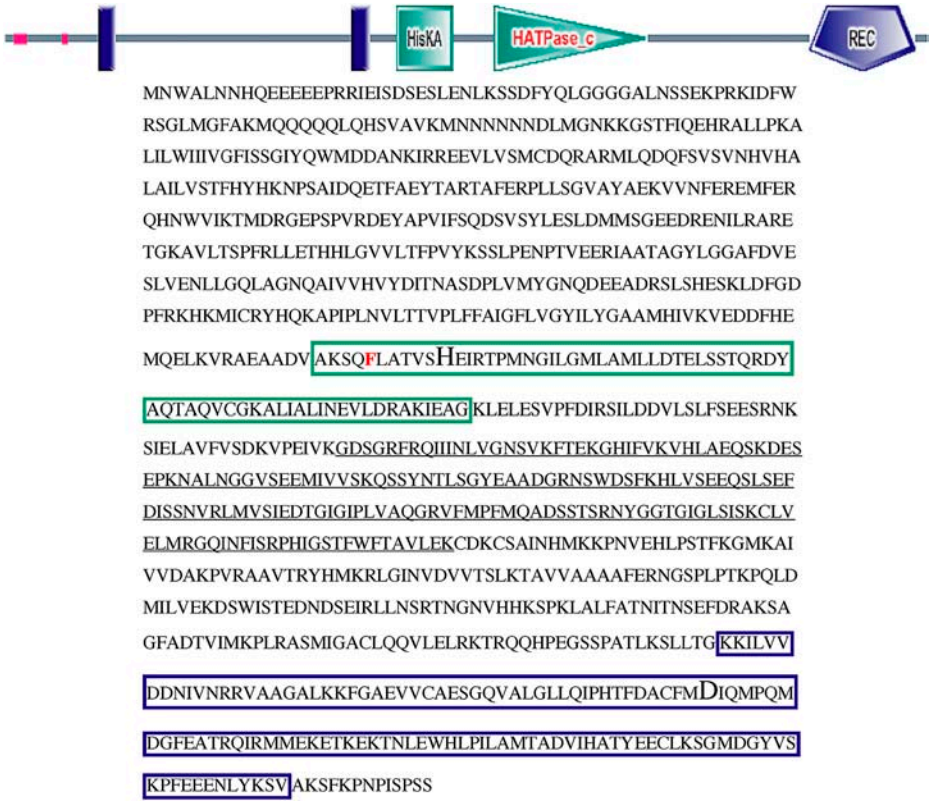

Figure 5. Location of the CRE1 mutation in habituated calli. Schematic of the CRE1 protein domains was generated from the amino acid sequence at http://smart.embl-heidelberg.de/. Blue rectangles, Transmembrane domains. Green square/green box, HK domain. Green triangle/black underscoring, Catalytic domain. Blue pentagon/blue box, RR domain. The conserved His and Asp residues within the HK and RR domains, respectively, are depicted by a larger font size. The location of the amino acid change in habituated calli (F453L) is shown in red.

(approximately 3-fold induction after 3 or 6 d on cytokinin-rich media [Che et al., 2002]). These results suggest that cytokinin sensitivity may be modulated through regulation of cytokinin-receptor production. Notably, the expression of *CRE1* was not up-regulated in nonhabituated callus cultures maintained in the presence of cytokinin for 6 weeks (Table V).

Another member of the cytokinin-receptor family, *AHK3*, displayed altered expression in habituated calli. In this case, the cytokinin receptor was down-regulated 3.6-fold. Perhaps this down-regulation is a response to the overabundance of the *CRE1* cytokinin receptor.

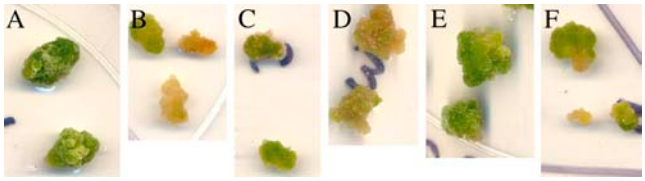

Figure 6. Isolation of habituated callus lines (Hab1–Hab4) from wild-type Col root segments after four successive passages onto media containing both auxin (3,000 ng/mL 1-NAA) and cytokinin (300 ng/mL BA), followed by seven successive passages of the same callus cultures onto media either containing or lacking cytokinin. A, Hab1 maintained in the absence of BA. B, Hab1 maintained in the presence of BA. C, Hab3 maintained in the absence of BA. D, Hab3 maintained in the presence of BA. E, Hab4 maintained in the absence of BA. F, Hab4 maintained in the presence of BA.

AHK2 levels were down-regulated by only 1.7-fold in habituated calli. The *AHK2* cytokinin receptor has been shown to play a very minor role in callus proliferation, as demonstrated by the wild-type callus induction of *ahk2* mutant tissues, as well as by the severely compromised callus induction of *cre1 ahk3* double mutant tissues (Higuchi et al., 2004). The *CKI2* HK, the only HK in Arabidopsis lacking a putative receptor domain, was down-regulated 2.4-fold in habituated callus tissue. This gene was originally implicated in cytokinin signaling, as overexpression of *CKI2* confers habituation on Arabidopsis callus cultures (Kakimoto, 1996). However, no subsequent evidence that CKI2 is involved in cytokinin signaling has been reported to date, as null mutations of *CKI2* have no obvious cytokinin-related phenotype (Higuchi et al., 2004).

Expression of the HK *AtHK1* was up-regulated 3.2-fold in habituated callus tissues. Up-regulation of *AtHK1* in response to cytokinin has been reported for callus tissue, after 3 d on cytokinin-rich media (Che et al., 2002), as well as seedling tissue, after a 1.5 h exposure to cytokinin (D.J. Somers, personal communication). AtHK1 has been implicated in osmosensing, and the *AtHK1* transcript is induced by both high (e.g. NaCl) and low (e.g. distilled water) osmolarity (Urao et al., 1999). It was recently shown that SLN1, a related osmosensor in yeast, responds specifically to a change in turgor (Reiser et al., 2003). T87 cell cultures are

rapidly dividing with respect to freshly derived callus cultures and also have a distinct morphology. For example, cells within T87 callus cultures are more friable in texture and vitreous in appearance than cells within freshly derived callus cultures. Perhaps up-regulation of *AtHK1* in the rapidly dividing T87 calli reflects changes in turgor and cell wall adhesion within the habituated cell line.

An analysis of the expression levels of RRs in habituated calli revealed up-regulation of the Type-A RRs *ARR7* and *ARR15*. These proteins are primary cytokinin-response genes (D'Agostino et al., 2000) and act as negative regulators of cytokinin signaling (Hwang and Sheen, 2001; To et al., 2004). For example, several higher-order Type-A RR mutant combinations display a cytokinin-hypersensitive phenotype in callus culture and root elongation (To et al., 2004). Thus, inactivating these proteins in vivo relieves their repressive action on cytokinin signaling. Cytokinin induction of *ARR15* in *cre1* rosette leaves is wild type (Kiba et al., 2002; Nishimura et al., 2004). However, cytokinin induction of *ARR15* is specifically impaired in root tissues of a *CRE1* mutant (*cre1-1*; Kiba et al., 2002) and is completely abrogated in root tissues of the *cre1 ahk2 ahk3* triple cytokinin-receptor mutant (Higuchi et al., 2004), indicating that this Type-A RR functions downstream of the CRE1 cytokinin receptor.

Whereas expression of the Type-B RRs in habituated calli was relatively unchanged (using a 2-fold-change cutoff), expression of two pseudo RR genes was altered in T87 calli. Expression of *APRR2* was down-regulated 3.0-fold, while that of *APRR5* was up-regulated 2.1-fold. The pseudo RRs bear homology to the Type-A and Type-B RRs, yet lack the conserved Asp residue required for propagation of a His-Asp phosphorelay. They fall into two broad categories (Makino et al., 2000): those possessing a C-terminal motif similar to that found in the CONSTANS gene product, including *APRR5*, and those lacking this C-terminal motif, including *APRR2*. This C-terminal motif seems to confer circadian regulation of transcription on the pseudo RRs (Yamamoto et al., 2003). Recently, reports that cytokinins, among other phytohormones, are involved in circadian rhythms have emerged (Hanano et al., 2005). Perhaps *APRR5* represents a point of cross talk between cytokinin signaling and circadian rhythms.

Given that *APRR2* belongs to a gene family that has been implicated in phytochrome-mediated circadian regulation yet is not itself regulated by light, it is intriguing that the *APRR2* transcript is down-regulated in habituated callus tissue. *APRR2* possesses a C-terminal protein domain that is homologous to the DNA-binding domain typical to Type-B RRs and thus may serve as a transcription factor (Makino et al., 2000). Although APRR2 cannot itself propagate a phosphorelay, protein interactions between RRs have been reported (Nakashima et al., 1991; Baikalov et al., 1998; Müller-Dieckmann et al., 1999). Perhaps APRR2 represents a point of cross talk between light- and cytokinin-mediated signaling pathways, as has been suggested for the Type-A RR ARR4. The *ARR4* transcript accumulates in response to cytokinin (D'Agostino et al., 2000), while the protein accumulates in response to red light (Sweere et al., 2001). Furthermore, the ARR4 protein has been shown to bind to PHYTOCHROME B (PHYB), stabilizing this light receptor in its active form (Sweere et al., 2001). The fold-change of *ARR4* expression in habituated calli compared to freshly derived calli was +1.9. Although PHYB levels were unaltered in habituated calli, one of the five red light receptors in Arabidopsis, *PHYA*, was down-regulated 3.6-fold in habituated callus cultures.

The HPts *AHP1* and *AHP5* were down-regulated in habituated calli by 12.5-fold and 1.9-fold, respectively. Overexpression of *AHP2* confers cytokinin hypersensitivity on both root and hypocotyl tissues of transgenic plants (Suzuki et al., 2002), demonstrating a positive/stimulatory role for an HPt protein in cytokinin signaling. In addition, AHP1 and AHP2 have been shown to translocate from the cytoplasm to the nucleus in a cytokinin-dependent manner (Hwang and Sheen, 2001). Furthermore, coexpression of AHP1, 2, 3, or 5 along with CRE1, in a heterologous system, demonstrates that these HPt proteins are able to accept and titrate out the CRE1-initiated signal, albeit to varying degrees (Suzuki et al., 2001).

While we cannot rule out the possibility that T87 habituated cell line-derived callus cultures produce higher levels of cytokinin than nonhabituated callus cultures, the results of this study do not support this hypothesis for a number of reasons. First, several known cytokinin-inducible genes are down-regulated in habituated callus cultures rather than up-regulated (Table V). In addition, we found no up-regulation of cytokinin-producing enzymes or down-regulation of cytokinin-degrading enzymes. In fact, we saw an up-regulation of cytokinin-degrading enzymes (Table V). Furthermore, we found that a large percentage of genes up-regulated in nonhabituated callus cultures in response to the presence of cytokinin are actually down-regulated in habituated callus cultures (Supplemental Table III). Based on our results, it seems less likely that habituation is caused by an overproduction of cytokinins by the callus tissue and more likely that habituation is caused by altered expression of one or more cytokinin-signaling genes, for example, the cytokinin receptor *CRE1*.

We verified the 19-fold up-regulation of CRE1 in habituated callus cultures at both the mRNA and protein level. It is possible that overexpression of this cytokinin receptor alone is responsible for habituation in the T87 cell line. Overexpression of CRE1 may allow habituated T87 cells to sense very low endogenous levels of cytokinin. Other possible explanations for CRE1 overexpression-induced habituation include a high-enough concentration of CRE1 protein in the plasma membrane to initiate cytokinin-related signaling events in the absence of cytokinin, or promiscuous interactions between CRE1 protein molecules and other proteins. The mechanism for the inappropriate

expression of *CRE1* in habituated calli, however, remains to be seen. Perhaps aberrant expression of CRE1 is due to a mutation in the gene or promoter sequence of *CRE1*. To explore this possibility, we sequenced 19,742 bases from habituated calli, corresponding to the promoter and gene sequences for *AHP1*, *TMK3*, and *CRE1*. Our results revealed only one nucleotide change within the *CRE1* gene sequence, which corresponds to a substitution of a Phe residue for a Leu residue within the HK domain of the protein sequence. Another study based on citrus callus cultures did not find DNA sequence variations in calli over time (Hao et al., 2004), suggesting that while single base-pair changes have been documented during the culture process (Phillips et al., 1994), the DNA in callus tissue is not necessarily undergoing large-scale genome-wide mutagenesis, which could account for aberrant expression of many gene products.

Experimental evidence indicates that CRE1 possesses both kinase and phosphatase activities (T. Kakimoto and Y. Helariutta, personal communication). Point mutations within a related bacterial HK, EnvZ, have been identified that affect the kinase activity, the phosphatase activity, or both enzymatic activities of this bifunctional enzyme. Based on current work characterizing the effects of point mutations on the enzymatic activity of EnvZ (Dutta and Inouye, 1996; Hsing et al., 1998; Dutta et al., 2000), however, it is not possible to predict how an amino acid substitution of a Phe residue for Leu 453 would affect CRE1 expression or function in vivo. Examination of the sequencing chromatograms revealed that the single base change detected in the *CRE1* coding sequence was found in a homozygous rather than heterozygous state (data not shown). Because callus tissues do not undergo meiosis, the simplest explanation for the existence of this mutation in the homozygous state is that it was present in the original plant from which the T87 cell line was derived. If not due to a mutation, perhaps aberrant expression of CRE1 is due to an epigenetic change at the *CRE1* locus.

Up-regulation of several transposon-related elements and DNA- and chromatin-modifying enzymes in habituated calli make the hypothesis that *CRE1* overexpression is due to epigenetic changes at the *CRE1* locus an attractive one. The relationship between DNA and chromatin modification, and gene expression, has received much attention over the last several years (for review see Loidl, 2003). Thus, we now know that DNA hypermethylation is correlated with repression of gene expression, while DNA hypomethylation is correlated with up-regulation of gene expression. Similarly, hyperacetylation of histones is associated with euchromatin, while hypoacetylation is associated with heterochromatin. Furthermore, particular patterns of Lys methylation on nucleosome components are associated with either silenced or expressed genes.

In addition to the many different kinds of mutations that have been documented during the tissue culture process (e.g. chromosomal translocations, inversions, deletions, duplications, and base-pair changes; Phillips et al., 1994), variations in DNA methylation patterns (both hypermethylation and hypomethylation) among callus cultures have been identified in many cases as well (Phillips et al., 1994; Kaeppler et al., 2000). These alterations in DNA methylation are retained by plants regenerated from the callus tissues, as well as by the progeny of those plants. Particular types of histone and DNA modifications are often associated with transposable elements, e.g. methylation of Lys-9 on Histone 3, and methylation of DNA. When these modifications are lost or changed by specific mutations such as those in *DDM1* or by continued passage in culture, both enhanced activation of transposons, and increased transcription of transposon-related elements is seen (Planckaert and Walbot, 1989; Kaeppler et al., 2000; Lippman et al., 2004). The mechanism for targeting particular regions of the genome for silencing via DNA and histone modifications is most likely mediated by RNA interference (Bender, 2004; Lippman and Martienssen, 2004). Interestingly, one of the DICER-like genes in Arabidopsis (*DCL3*, At3g43920) is up-regulated 2.5-fold in habituated calli (Table IX). In addition to the support provided by this transcriptome profile of a habituated cell line, a direct link between methylation and a cytokinin-dependent developmental process was identified in Petunia (Prakash et al., 2003). In this case, shoot-induction of cultured Petunia leaf discs was inhibited by the methylation-inhibiting drugs AcaC and AzadC. This inhibition of a cytokinin-dependent process was coupled with a decrease in cytosine methylation of the culture tissues.

The overexpression of cytosine methyltransferases, histone methyltransferases, histone deacetylases, and chromatin remodeling factors, in habituated callus cultures, indicates that the chromatin in T87 cells is under a more dynamic state of regulation than the chromatin in nonhabituated callus cultures. The overexpression of DNA methylation enzymes and histone deacetylases, in particular, would suggest that T87 cells are investing in processes leading to silencing of gene expression. Yet, we see indications of inappropriate overexpression of transcripts in T87 cells, e.g. many transposon-related elements, as well as *FWA*. These alterations in gene expression indicate that aberrant activation of transcription is occurring in habituated calli. How can these two opposing activities be reconciled? One possible explanation is that heritable, epigenetic changes occur during the process of habituation, leading to a default expression state of global up-regulation. T87 cells may then induce expression of silencing machinery to actively repress specific genes. These targets for gene silencing would presumably be genes whose expression would reduce the proliferation rate of habituated calli, confer a dependence on environmental cues for cell growth and division, and/or divert energy and resources toward processes nonessential for the maintenance of habituated callus cultures.

Several strategies exist for investigating epigenetic changes within a plant tissue sample: global

quantification of methylcytosine levels, Southern-blot analysis of genomic DNA cleaved with methylation-sensitive restriction enzymes, genomic bisulfite sequencing, and methylation-sensitive PCR. A scan of the genomic region between the *CRE1* start codon and the nearest upstream gene, using GeneQuest 5.52 (DNASTAR), revealed 82 CpG sites, 53 CpNpG (N = A, T, C, or G) sites, and 654 CpHpH (H = A, T, or C) sites. Future work characterizing DNA methylation patterns associated with specific genes in habituated callus cultures, namely, *CRE1*, could be important for elucidating the mechanism of habituation in the T87 cell line.

CONCLUSION

Habituated callus tissues can be isolated naturally from several different species of plants (e.g. tobacco [Meins and Foster, 1986], sunflower [*Helianthus annuus*; Boeken et al., 1974], Arabidopsis [Axelos et al., 1992]) and from many different tissues within the same plant (e.g. root, stem, leaf, hypocotyl, cotyledon, embryos). All that is required is time, that is, continued passage in tissue culture. Overexpression of specific cytokinin-signaling components has been shown to artificially confer habituation on callus tissues (Kakimoto, 1996; Hwang and Sheen, 2001; Sakai et al., 2001; Osakabe et al., 2002). Our study, however, identifies a specific gene whose overexpression may account for the naturally occurring phenomenon of habituation, the cytokinin receptor *CRE1*. This result is particularly interesting since the transcriptome profiling data of habituated and nonhabituated callus cultures argue against the hypothesis that habituation is caused by an overproduction of cytokinins.

There are reports that epigenetic changes do occur as a result of the plant cell culture process and that DNA methylation patterns are highly variable among plants regenerated from cultured tissues. Our microarray data indicates that the chromatin in habituated calli is under a more dynamic state of regulation than the chromatin in nonhabituated calli. For example, we see up-regulation of several DNA- and chromatin-modifying enzymes, the methylation-regulated gene *FWA*, and several transposon-related elements in habituated calli. Our microarray results point to epigenetic modification as a mechanism for habituation. Many possible genes, including *CRE1*, could be epigenetically modified in planta whose overexpression or underexpression may lead to callus habituation.

Interestingly, a particular locus (*Hl-2*) regulating the cytokinin dependence of callus cultures induced from leaf tissues has been identified in tobacco (Meins and Foster, 1986). Experimental evidence is consistent with an epigenetic change at the *Hl-2* locus conferring a meiotically transmissible, yet reversible, habituated state on tobacco leaf explants (Meins and Thomas, 2003). As homologs for the *CRE1* HK and several of the other His-Asp phosphorelay proteins (HPts and RRs) involved in cytokinin signaling have been identified in a variety of plant species (Sakakibara et al., 1998, 1999; Papon et al., 2003; Qiu-min et al., 2004; Yonekura-Sakakibara et al., 2004), it is tempting to speculate that the *Hl-2* locus encodes a member of the cytokinin signal transduction pathway.

MATERIALS AND METHODS

Callus Growth Conditions

Sterilized seeds of Arabidopsis (*Arabidopsis thaliana*), ecotype Col, were germinated on media, pH 5.7, containing full-strength Murashige and Skoog (MS; Murashige and Skoog, 1962), 0.05% (w/v) MES, 1% (w/v) Suc, 1/1,000 volume of Gamborg's vitamin solution (Sigma), and 0.8% (w/v) washed agar (MS + Gamborg). Plated seeds were cold treated for ≥2 d at 4°C, exposed to red light for 20 min to synchronize germination, and maintained in a vertical orientation at 23°C, under constant illumination (42 μmol m^{-2} s^{-1}), for 2 to 3 weeks. For callus induction, approximately 5-mm root segments were transferred to fresh MS + Gamborg media supplemented with 3,000 ng/mL α-naphthaleneacetic acid (1-NAA) and 300 ng/mL 6-benzylaminopurine (BA; Sigma). After 6 weeks in culture, the resultant callus tissue was passed onto fresh MS + Gamborg media supplemented with 3,000 ng/mL 1-NAA, and either 0 (−BA) or 300 (+BA) ng/mL BA, for an additional 6-week time period. RNA was collected following this second 6-week incubation. Calli derived in this way were referred to as freshly derived calli, or FC.

An aliquot of the T87 cell line was obtained from Sebastian Bednarek (University of Wisconsin, Madison). T87 calli were maintained on MS + Gamborg media supplemented with 3,000 ng/mL 1-NAA and passed onto fresh media at 3-week intervals. Passages were carried out at 3-week rather than 6-week intervals due to the rapid proliferation of T87 calli compared to FC calli. Prior to RNA isolation, T87 calli were passed onto fresh media supplemented with either 0 (−BA) or 300 (+BA) ng/mL BA for an additional 3-week period. RNA was collected following this second 3-week incubation.

For surface area measurements, calli outlines were traced on the bottom of the petri dish at the time of passage as well as after 3 (T87 calli) or 6 (FC calli) weeks. Scanned TIFF files of these petri dishes were opened in NIH Image, and the surface area at the beginning and ending points was calculated by tracing each outline with a mouse pen.

Isolation of New Habituated Cell Lines

Callus cultures were initiated from wild-type Col roots and maintained in culture as previously described for freshly derived callus cultures. After four passages, callus cultures were numbered and passed onto media either containing or lacking the cytokinin BA. At 6-week time intervals, cultures were passed onto fresh media with the same hormone content. After seven passages, four candidate habituated cell lines were identified, named Hab1 to Hab4.

RNA Isolation and RT-PCR

Callus tissue was frozen in liquid nitrogen and disrupted using a mortar and pestle. Total RNA was isolated from approximately 100-mg quantities of ground tissue using the RNeasy Plant Mini kit (Qiagen). Reverse transcription was carried out on 2 μg of total RNA with the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen) at 42°C. Two microliters of prepared cDNA, or dilutions of prepared cDNA, were used for a 40-cycle PCR. The primers used for amplification of several transcripts were as follows: *CRE1*, 5′-tcatcaagaagaagaagaagaagccacgaa-3′ and 5′-ccttctggatgttgttgtcttgtttt-3′; *AtHK1*, 5′-aggaaggtgttcgataaaatgactgaatg-3′ and 5′-caatgaagtttggaatgctgatggtttag-3′; *FWA*, 5′-gagatagaccagttcaattccagatacct-3′ and 5′-tctccacctgaattttctgccacttgtc-3′; *TMK3*, 5′-ggcgacacctttaacctcctccactctg-3′ and 5′-cgtcgtcgaggctgatgtttgggtcta-3′; *ARR5*, 5′-aggttttgcgtcccgagatgttagata-3′ and 5′-gattactctatgcctgggatgactggat-3′; *AHP1*, 5′-tcttagaagggattttggacagc-3′ and 5′-agaagctgcggaagacaacacaag-3′; and *CKX3*, 5′-tctaggacctgtttatatgttgacg-3′ and 5′-aacccccatattagcatcctcac-3′.

Microarray Analysis

Four biological replicates of RNA were prepared from each of the following tissue types: habituated T87 callus grown in the presence of cytokinin (T87 + BA), habituated T87 callus grown in the absence of cytokinin (T87 − BA),

freshly derived callus grown in the presence of cytokinin (FC + BA), and freshly derived callus grown in the absence of cytokinin (FC − BA). cDNA and cRNA preparation from total RNA, labeling and fragmentation of the cRNA, and hybridization to the Arabidopsis 60mer microarray were carried out by NimbleGen Systems. Gene expression data (corresponding to approximately 28,500 locus identifiers) were analyzed following quantile normalization and robust multiarray averaging (Irizarry et al., 2003). The average signal intensity for a given gene was considered significantly different between treatments if a SAM analysis on The Institute for Genomic Research (TIGR) MultiExperiment Viewer (available at http://www.tm4.org/mev.html) returned the gene in ≥5/11 runs. For SAM initialization, the following parameters were used: number of permutations, 100; SO parameters, Tusher et al. (2001) method; q values, not calculated; imputation method, K-nearest neighbors imputer; number of neighbors, 10; and delta value, the largest value estimated to return a 0% false discovery rate.

qPCR Analysis

RNA was isolated as described above. After isolation, 5 μg of total RNA was treated with RQ1 DNase (Promega) per the manufacturer's instructions. Two micrograms of DNase-treated RNA was used for a reverse transcription reaction as described above. qPCR was carried out on an iCycler (Bio-Rad) in 20-μL reaction volumes with the following program: 95.0°C for 10 s; 95.0°C for 10 s, 59°C or 60.5°C for 45 s, 45×; 95.0°C for 1 min; 55.0°C for 1 min; and 55.0°C for 10 s, repeat 80× increasing setpoint temperature by 0.5°C each cycle. The primers used for amplification of several transcripts were as follows: *CRE1*, 5′-attgatcaggagacatttgc-3′ and 5′-ggctctcctctatccattgtc-3′; *AHK2*, 5′-gtaatcttgaaccgattttacagca-3′ and 5′-accaaggattagacaccaccat-3′; *AHK3*, 5′-tctgggaaagaagatcgtgaa-3′ and 5′-ccgagatacccgttagtagcct-3′; *TMK3*, 5′-gaatacgcagtgacgggaa-3′ and 5′-tctagggctttacgaccagtg-3′; *FWA*, 5′-attagtccaggattgtctgcaa-3′ and 5′-acctgaattttctgccacttgt-3′; *AtHK1*, 5′-ctttgagcaagctgatccttctaccactc-3′ and 5′-caagtttcgcacaatacatagtccaagtc-3′; *AHP1*, 5′-caaaggtagcagctccagt-3′ and 5′-gctccagcttgaacagagt-3′; and *ACT2* (control), 5′-gcatgaagatcaaggtggttgcac-3′ and 5′-atggacctgactcatcgtactcact-3′. Primers were selected to amplify products less than 200 bases long, to contain a G/C content of 30% to 60%, and to have a melting temperature of 55°C to 65°C. In all cases, at least one primer from each pair spanned an intron. After program completion, the products were visualized on a 2% agarose gel. Product size determination and melting curve analysis were used to eliminate aberrant products from the analysis.

DNA Isolation and Sequencing

Genomic DNA was isolated from approximately 100-mg quantities of ground callus tissue using the DNeasy Plant Mini kit (Qiagen). Sequencing of the *CRE1*, *TMK3*, and *AHP1* promoter and coding regions was carried out on genomic DNA regions amplified with Ex-Taq DNA polymerase (Takara Mirus Bio), with BigDye Terminator version 3.1 (Applied Biosystems). The following cycling parameters were used: 96° for 2 min, followed by 28 cycles of 94° for 15 s/60° for 3 min 45 s. Sequencing reactions were purified with the CleanSEQ reaction clean-up kit (Agencourt) and analyzed by the DNA Sequencing facility at the University of Wisconsin, Madison (http://www.biotech.wisc.edu/ServicesResearch/DNA/DNASeq). Promoter and coding regions were determined using the SeqViewer tool on the TAIR Web site (http://www.arabidopsis.org/servlets/sv). Promoter regions were defined as the regions upstream of the 5′ untranslated region (UTR) of the gene of interest and downstream of the 3′ UTR of the nearest upstream gene. Coding regions were defined as the sequences between and including the 5′ and 3′ UTRs for each gene. Appropriate primers for amplification and sequencing were selected using PrimerSelect 5.52 (DNASTAR).

Isolation of Microsomes

Approximately 25 g of either T87 or freshly derived callus tissue was flash frozen in liquid nitrogen, and the resulting frozen tissue was mixed with 3 mL of grinding buffer (290 mM Suc, 250 mM Tris-HCl, pH 7.6, 25 mM EDTA, 25 mM sodium fluoride, 50 mM sodium pyrophosphate, 1 mM ammonium molybdate, 0.5% (w/v) polyvinylpyrrolidone, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 μg/mL pepstatin, 1 μg/mL E64, 1 μM bestatin, and 100 μM 1,10-phenanthroline) per gram of tissue. The mixture was homogenized in stages, first using a kitchen blender and second using a Polytron. The suspension was filtered through four layers of Miracloth and centrifuged for 5 min at 1,500g. The supernatant was collected and centrifuged for 60 min at 100,000g, and the pellet was resuspended in resuspension buffer (50 mM ammonium bicarbonate, 20 mM sodium fluoride, 1 mM dithiothreitol, 1 mM ammonium molybdate) and homogenized in a Potter grinder. The suspension was then centrifuged for 5 min at 13,200 rpm and resuspended in 100 mM sodium carbonate, pH 11.00. After 90 min on ice, this suspension was centrifuged for 5 min at 13,200 rpm and resuspended in resuspension buffer. Methanol was added to a final concentration of 60% (v/v), and sequencing-grade modified trypsin (Promega) was added to 2.5% of the total protein concentration as measured by the bicinchoninic acid method (Pierce Biotechnology). After an overnight incubation at 37°C, another 2.5% aliquot of trypsin was added, and the mixture was allowed to remain at 37°C for an additional 5 h. The mixtures were then frozen at −20°C, thawed, and the excess methanol was evaporated under vacuum. Formic acid was added to the solution at a concentration of 1% (v/v), and the mixture was centrifuged to pellet any debris. The supernatant was then extracted using Varian SPEC PT C18 solid-phase extraction pipette tips, and eluted using 90% (v/v) acetonitrile/0.1% (v/v) formic acid. The acetonitrile was evaporated under vacuum.

AQUA Experiments

The peptide SSL-PEN-PTV-EER, corresponding to a tryptic peptide within the external loop of CRE1, was synthesized using PIN peptide synthesis techniques, incorporating $^{13}C_6$,^{15}N-Leu as an isotopic label (Peptide Synthesis Facility, University of Wisconsin, Madison; http://www.biotech.wisc.edu/ServicesResearch/Peptide/PeptideSynth/). A 1.5 pmol/μL solution was prepared by serial dilutions from a 2 mg/mL stock and was used as an internal standard for all AQUA experiments. Its mass spectrometry and tandem-mass spectrometry spectra were measured on an API 365 triple quadrupole mass spectrometer, and its y_9 (2+) ion (683.2/536.2 labeled; 679.5/536.2 unlabeled) was chosen for selected ion monitoring on the basis of its strong signal.

Ten picomoles of the stable isotope-labeled internal standard peptide was added to 45 μg total protein, and the resulting solution was diluted to a volume of 60 μL. Online liquid chromatography separation was performed using a Vydac C18 HPLC column (1 mm i.d. by 15 cm length). Peptide elution was performed with a gradient from 0.05% TFA in water to 20% acetonitrile/0.05% TFA over 50 min, followed by a gradient to 90% acetonitrile/0.05% TFA over 50 min. The y_9 (2+) ions corresponding to both the labeled internal standard and the native peptide tryptic fragment were monitored via single reaction monitoring. Extracted ion chromatograms were integrated, and the abundance of the native peptide was calculated based on comparison with the internal standard peptide. The T87 and freshly derived callus samples were each examined five times, and the results were averaged to determine the abundance of CRE1 in each sample.

Microarray data from this article can be found as supplemental data.

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