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Involvement of Arabidopsis Histone Acetyltransferase HAC Family Genes in the Ethylene Signaling Pathway

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Epigenetic modifications play a fundamental role in regulating chromatin dynamics and gene expression. The level of histone acetylation is controlled by two functionally antagonistic enzymes, namely histone acetyltransferase (HAT) and histone deacetylase (HDAC). CREB-binding protein (CBP)/p300 proteins, a subfamily of highly conserved HATs, are involved in various physiological events including proliferation, differentiation and apoptosis. In this work, we study the poorly known function of their homologous genes, the HAC genes, in Arabidopsis. We found that hac1-involved mutants displayed pleiotropic phenotypes, in particular hypersensitivity to ethylene both in the dark and in the light. We also found that the transcriptional levels of ethylene-responsive genes are significantly higher in the hac1hac5 double mutant than in wild-type plants. Moreover, an ethylene synthesis inhibitor cannot release the triple responses of hac mutants. These results suggest that HACs are involved in the ethylene signaling pathway.

Keywords: HAC family genes • Histone acetyltransferase.

Abbreviations: ACC, 1-aminocyclopropane-1-carboxylic acid; AVG, aminoethoxyvinylglycine; CBP, CREB-binding protein; CTR1, CONSTITUTIVE TRIPLE RESPONSE 1; EIN3, ETHYLENE INSENSITIVE 3; ER, endoplasmic reticulum; ERF, ethylene response factor; FLC, FLOWERING LOCUS C; GUS, β-glucuronidase; HAC, histone acetyltransferase of the CBP family; HAT, histone acetyltransferase; HDAC, histone deacetylase; LNM, low nutrient medium; MS, Murashige and Skoog; PP2A, protein phosphatase 2A.

Introduction

In recent years, it is widely accepted that epigenetic regulations as well as genetic regulations play a critical role in eukaryote growth and development. Epigenetic traits are the stably heritable phenotypes resulting from changes in chromatin structure rather than in the DNA sequence (Bird 2007, Berger et al. 2009, Tarakhovsky 2010). Epigenetic modifications include covalent modifications, occurring directly on DNA or on the chromatin, and the replacement of histone variants in the core histones of the nucleosome, which play a key role in regulating chromatin dynamics and gene expression (Strahl and Allis 2000, MacDonald and Howe 2009). The histone acetylation levels are controlled by two functionally antagonistic enzymes, namely histone acetyltransferase (HAT) and histone deacetylase (HDAC). HATs neutralize positive charges in the histone tail and thus decrease the interactions between histone and DNA or between histone and histone (Hong et al. 1993, Fletcher and Hansen 1996, Steger and Workman 1996, Luguer and Richmond 1998). The structure of chromatin is modified in terms of weakened interactions, so that transcriptsome can more easily access the target and subsequently induce the gene’s expression (Norton et al. 1989)

In mammals, CREB-binding protein (CBP)/p300 is an important subfamily of HATs, which are involved in a wide array of critical cellular activities, such as DNA repair, cell growth, cell differentiation and apoptosis (Stein et al. 1990, Chirvia et al. 1993, Eckner et al. 1994, Giles et al. 1998, Giordano and Avantaggiati 1999, Karamouzis et al. 2007). CBP/p300 generally act as transcriptional co-activators in the regulation of gene expression (Goodman and Smolik 2000, Das et al. 2009, Wang et al. 2010). For instance, in Drosophila, CBP-mediated acetylation of histone H3K27 can antagonize the silencing effect of the Polycomb gene-induced trimethylation of histone H3K27 (Tie et al. 2009). CBP/p300 can also acetylate non-histone proteins, such as p53 (Grossman 2001, Ito et al. 2001) or c-Myb (Faiola et al. 2005).

In Arabidopsis, CBP/p300-like genes (HAC genes) have been found to regulate flowering time. The transcript level of FLOWERING LOCUS C (FLC) was significantly higher in hac1-involved mutants than in wild-type plants. HAC family genes were proved to regulate flowering time through the autonomous pathway; however, no variations have been detected in the expression level of FLC repressors and activators. Moreover, chromatin immunoprecipitation assay of the three regions of
the FLC locus did not show any changes in H3 acetylation. Since the HDAC inhibitor trichostatin could decrease the elevated FLC expression level in hac mutants, one possible mechanism could be that HACs directly acetylate FLC itself (Deng et al. 2007, Han et al. 2007).

Ethylene is an important gaseous plant hormone, which modulates various growth dynamics and developmental events, such as seed germination, seedling growth, fruit ripening and organ senescence (Yoo and Sheen 2008, Stepanova and Alonso 2009, Yoo et al. 2009). In the condition of a low ethylene level, inactivated ethylene receptors directly interact with a negative regulator, CONSTITUTIVE TRIPLE RESPONSE 1 (CTR1), to repress the downstream signaling (Bleecker et al. 1988, Hua and Meyerowitz 1997, Wang et al. 2006, Xie et al. 2006). In the condition of a high ethylene level, binding of ethylene disassociates the receptor complex, which releases CTR1 from the endoplasmic reticulum (ER) membrane and in turn de-repress the EIN2-mediated downstream ethylene signaling pathway (Kieber et al. 1993, Clark et al. 1998, Alonso et al. 1999, Huang et al. 2003, Bisson et al. 2009). In the nucleus, an ETHYLENE INSENSITIVE 3 (EIN3) family of transcriptional activators, finely regulated by the SCF EBF1/EBF2–26S proteasome pathway, activates the expression of ethylene-responsive genes (Chao et al. 1997, Solano et al. 1998, Guo and Ecker 2003, Potuschak et al. 2003, Gagne et al. 2004). EIN2 plays an unknown role in promoting the proteasomal degradation of EBF1/2 proteins, which send the ethylene signal from the ER to the nucleus (An et al. 2010).

Similar to CBP/p300 in mammals, the HAC family genes play critical and pleiotropic roles in plant growth and development. However, our understanding of the function of HACs is limited to the flowering time control pathway. In this work, we investigated the function of the HAC family genes in the ethylene pathway. We found that hac1-involved mutants were hypersensitive to ethylene and that they influenced the expression levels of ethylene-responsive genes. We also systematically studied the morphological phenotypes of hac mutants. Our results shed some light on the future study of the multiple functions of HACs.

Results

Characterization of T-DNA insertional mutants of the Arabidopsis HAC family genes

There are five members in the HAC family, namely HAC1, HAC2, HAC4, HAC5 and HAC12. All the HAC members share the CBP-type HAT domain; however, the HAT domain of HAC1 but not that of HAC2 possesses acetyltransferase activity, while the situation had not been tested in the other HAC members (Pandey et al. 2002). With the exception of HAC2, the HAC members also share the partially conserved KIX domain, which is the CREB-binding domain; PHD, a partially conserved plant homeodomain zinc finger; and two types of zinc finger domains ZnF-TAZ and ZnF-ZZ, which mediate protein–protein interactions in CBP/p300. From the ARBC seeds library, we found several T-DNA insertional mutants of HAC1, HAC4, HAC5 and HAC12 (Fig. 1A). For the HAC1 gene, we obtained four alleles, namely hac1-2, hac1-3, hac1-4 and hac1-6. The real-time PCR analysis showed that all four hac1 alleles are null mutants. All these alleles displayed late flowering, and dark green and wrinkly rosette leaves (Fig. 1C; Supplementary Fig. S1). Moreover, all the alleles except hac1-4 showed protruding gynoecia (Supplementary Fig. S1B).

To confirm that the phenotypes of hac1 mutants are caused by knockout of the HAC1 gene, we carried out a complementation experiment on the hac1 mutant by introducing two tagged HAC1s, pHAC1:FLAG-HAC1 and pHAC1:HA-HAC1, respectively, into the hac1-2 allele. In these two constructs, full-length HAC1 coding sequences (CDS) with an N-terminal FLAG or HA tag were driven by the HAC1 native promoter. In both transgenic plants, the morphological phenotypes, including late flowering, and dark green and wrinkly leaves, were rescued by the HAC1 gene. We also introduced pHAC1:FLAG-HAC1 and PHAC1:HA-HAC1 into wild-type plants. The transgenic plants showed similar phenotypes to the hac1 knock-out mutant, including protruding gynoecia and late flowering (Fig. 1C, E).

From the phylogenetic analysis of the HAT domain, HAC1 and HAC12 are closer to each other, while HAC4 and HAC5 are closer to each other. We obtained two null alleles of the hac12 mutant and found that they exhibited no obvious morphological phenotypes. The hac1hac12 double mutant exhibited enhanced morphological phenotypes compared with the hac1 single mutant (Fig. 1D). In addition, pHAC1:HA-HAC1 was able to rescue the phenotype of the hac1hac12 double mutant (Fig. 1E). We also obtained three alleles of the hac4 mutant and four alleles of the hac5 mutant, and neither the hac4 and hac5 single mutant nor the hac4hac5 double mutant showed any obvious phenotypes. We then carried out crosses to obtain all possible combinations of the hac mutants. We found that all the hac1-involved mutants showed similar and more severe phenotypes compared with the hac1 mutant, while other non-hac1-involved mutants showed no obvious phenotypes. Among all the mutants, the hac1hac5 double mutant displayed the most severe phenotypes and the hac1hac4hac5 mutant showed the second most severe phenotypes. Moreover, the hac1hac5 double mutant and the hac1hac5hac4 triple mutant are sterile. These results strongly indicate that HAC1, HAC12, HAC4 and HAC5 function synergistically, with HAC1 being dominant and HAC5 being the closest coordinator of HAC1.

Knowing that in mammalian cells CBP suppresses the expression of the MCP-1 and IL-8 genes in a dosage-dependent manner (Nettles et al. 2008), we introduced HAC1 into various mutant backgrounds to study the manner in which HAC1 is regulated. Using the flower organ phenotype as a standard to evaluate the effect of HAC1, we found that the regulation of HAC1 is dosage dependent. As shown in Fig. 1E, the pHAC1:HA-HAC1(WT) transgenic plant displays a protruding gynoecium phenotype, which is as severe as that of the hac1
Fig. 1 Characterization of T-DNA insertional mutants of the Arabidopsis HAC family genes. (A) Domain architecture of the HAC family proteins and the T-DNA insertion sites of corresponding hac mutants. Various domains are identified by different symbols and colors. HsCBP, human CBP; aa, amino acids; HAT, histone acetyltransferase; PHD, plant homeodomain zinc finger; KIX, CREB binding domain; ZnF-TAZ, transcriptional adaptor zinc finger; ZnF-ZZ, zinc finger present in dystrophin and CBP. The protein domains were predicted by Pandey et al. (2002) and the software ELM (http://www.elm.eu.org). The T-DNA insertion sites of all the hac alleles used in this work are marked at the corresponding positions of the translated protein products. (B) RT-PCR results show the deletion of the full-length HAC transcripts in the representative hac mutants. (C) Transformation of pHAC1::HA-HAC1 to the hac1-2 mutant can rescue the HAC1 mutation-caused late flowering, wrinkly leaf and
deletion mutant. Furthermore, 35 s:HAC1(hac1hac12) and 35 s:HAC1(WT) transgenic plants, driven by the constitutive promoter, show an even more severe protruding gynoecium phenotype compared with native promoter-driven transgenic plants (Fig. 1E). These results suggest that HAC1 regulates gene expression in a dosage-dependent manner.

**hac mutants are hypersensitive to ethylene**

To examine the multiple phenotypes induced by HAC mutations, we performed microarray analysis using the hac1hac12 double mutant. We found that several ethylene response factors (ERFs) were up-regulated in the hac1hac12 double mutant compared with the wild type. We subsequently confirmed the up-regulation of the ERFs in a series of hac mutants by Northern blot. The result showed that the transcriptional levels of ERF1, ERF4, ERF6 and ERF11 significantly increased in the hac1hac5 double mutant and increased at different levels in hac1 and hac1hac12 mutants compared with the wild type (Fig. 1A).

In the dark, ethylene induces the classical triple response, which is short stature, thickened hypocotyls and root, and an exaggerated hook. 1-Aminocyclopropane-1-carboxylic acid (ACC; Di Stefano et al. 2005) is the precursor of ethylene, which is applied to produce ethylene in plant growth medium. We found that the hac1hac5 double mutant and the hac1hac5hac4 triple mutant displayed a short hypocotyl and root on 0.5 Murashige and Skoog (MS) medium. On the medium supplemented with ACC, the hypocotyl and root in both mutants were even shorter, whereas the hac1hac5hac4 mutant displayed an exaggerated hook. The hypocotyl of the hac1hac5 mutant seems to be too short to form a hook. It has been found that copper acts as a cofactor of ethylene receptor 1 (ETR1) to bind ethylene, while silver competes with copper to bind ETR1, which affects its ability to bind to ethylene and in turn inhibits the ethylene-induced triple responses (McDaniel and Binder 2012). When the hac1hac5 and hac1hac5hac4 mutants were germinated on the 0.5 MS medium supplemented with ACC and AgNO₃, the short hypocotyls were significantly relieved and the hooks disappeared (Fig. 2B).

We then tested the ethylene response of all the hac mutants by dark growth assay. Without ACC, the hac mutants except hac1hac5 and hac1hac5hac4 showed no obvious phenotypes. Under treatment with a low concentration of ACC, the hac1-involved double and triple mutants displayed a shorter hypocotyl and root, and an exaggerated hook compared with the wild type. Under a high concentration of ACC, wild-type plants displayed obvious triple responses, while all the hac1-involved mutants displayed more severe phenotypes than the wild-type seedlings (Fig. 3A, B; Supplementary Fig. S2A).

It has been discovered that in the light ethylene hypersensitive mutants usually exhibit greener and longer hypocotyls.
compared with wild-type plants (Smalle et al. 1997). We tested the response of the hac mutants to ethylene in light conditions. On a low nutrient medium (LNM) plate, hac1hac5 and hac1hac4hac5 mutants showed longer and greener hypocotyls than wild-type seedling, which were enhanced on the ACC-supplemented medium. Other hac1-involved double and triple mutants also displayed longer and greener hypocotyls at different levels on the ACC-supplemented medium (Fig. 4A, B; Supplementary Fig. S2B). All these results suggest that the hac1-involved mutants are hypersensitive to ethylene.

Functional analysis of HAC genes in the ethylene pathway

Next, we studied the role of HACs in the ethylene synthesis pathway. In most plant tissues and cell types, ethylene can be produced from its precursor ACC by a series of enzymes. The ETHYLENE OVERPRODUCER 1 (ETO1) inhibits ACC synthase enzyme activity and targets it for degradation (Wang et al. 2002, Tsuchisaka and Theologis 2004). Aminoethoxyvinylglycine (AVG) is an ACC...
HAC family genes are involved in pleiotropic developmental processes

Besides late flowering and ethylene hypersensitivity, the hac1-involved mutants displayed pleiotropic developmental defects, including short primary root, dark green, wrinkly and indented leaves and protruding gynoecia (Fig. 6A, B, D). In particular, all the hac1-involved mutants showed a varied degree of fertility defects corresponding to other morphological phenotypes. Using the hac1hac5 double mutant as an example, we studied the development of the male and female gametophytes. When wild-type pollen was used to fertilize the mutant pistil, the seeds were not obtained. We then checked the mutant male gametophyte by 4,6-diamidino-2-phenylindole (DAPI) staining, which specifically stains nucleic acid. The male gametophyte of the hac1hac5 mutant is normal in the early developmental stage compared with the wild-type plant gametophyte. In the later developmental stages, two generative nuclei and one egg cell nucleus can be clearly detected in the wild-type male gametophyte, while the mutant male gametophyte appeared either as only one generative nucleus or as one generative nucleus and one egg nucleus (Fig. 6C). These results show that both the male and female gametophytes of the hac1hac5 mutant are aborted.

The relationship of the HAC family members

It has been shown in a previous study that HAC1, HAC5 and HAC12 are widely expressed in seedling and flower organs (Han et al. 2007). However, there are no data about HAC4. We used a histochemical β-glucuronidase (GUS) staining assay to detect the expression patterns of HAC genes. About 1,500 bp DNA sequences of HAC1, HAC12, HAC4 and HAC5 were used to drive the GUS expression. It turned out that all four HAC members showed similar expression patterns and that they were all widely expressed in the shoot meristem, leaf, hypocotyl, root, florescence and silique (Supplementary Fig. S3).

HAC5 and HAC12 mutation can strengthen all the HAC1 mutation-caused phenotypes, suggesting that HAC1, HAC5 and HAC12 function similarly. However, the involvement of hac4 weakened various phenotypes of the hac1hac5 mutant, such as triple responses, leaf size and petal length, suggesting a negative role for HAC4 against the other HACs. The protein sequence analysis showed that the HAT and KIX domains of the HAC4 protein are different from those of the other three genes, which might be the reason why it antagonizes the function of the other HAC proteins (Supplementary Fig. S4).

Discussion

Possible function of the HAC family genes in the ethylene signaling pathway

The Arabidopsis CBP/p300 HAT family has been found to regulate the flowering time control pathway. In the present work, we have shown that the HAC family is involved in the ethylene
signaling pathway. To identify the possible target of the HACs in the ethylene signaling pathway, we examined the expression level of \(EIN2\), \(EIN3\), \(CTR1\), \(EBF1\) and \(EBF2\) in the \(hac1hac5\) double mutant but found no significant changes in any of these genes.

To date, the details of the ethylene signaling pathway have not been well understood. In recent years, researchers have found some new components by genetic screening of the enhanced ethylene responses (Christians and Larsen 2007, Robles et al. 2007, Christians et al. 2008). The EERs are the negative regulators of ethylene signaling, which modulate ethylene signaling pathway in different ways. EER1 is one of the three protein phosphatase 2A (PP2A) regulatory subunits, which has been shown to associate with the kinase domain of \(CTR1\) and is required for the catalytic activity of PP2A (Larsen and Cancel 2003). EER3 encodes a prohibitin, AtPHB3, which has been shown by genetic analysis to be downstream of \(EIN2\) in the ethylene signaling pathway (Christians and Larsen 2007). EER4 is a TATA-box-binding TFIIID-associated transcription factor, which is a homolog of yeast TAF61. In yeast, TAF61 serves as a bridge between the transcription factor GCN4p and the TATA-binding protein. It either directly binds to the TATA-binding protein or binds to components of the SAGA complex (yeast Spt–Ada–Gcn5–acetyltransferase complex), which subsequently recruits the TATA-binding protein and RNA polymerase II for transcription of target genes (Natarajan et al. 1998). In Arabidopsis, EER4 interacts with EIN3, ERF1 and two PP2A catalytic subunits and is required for ethylene- and jasmonate-induced ERF1 expression (Robles et al. 2007). EER4 probably recruits the EIN3 and other transcription factors along with components of the TFIIID complex, and thus regulates the expression of a subset of genes required for either manifestation or subsequent dampening of the response to ethylene.

Since the \(hac1hac5ein3\) mutant showed similar morphological phenotypes to the \(hac1hac5\) mutant, we speculate that HACs might target EER4 or some other co-regulator of EER4. HACs might activate the expression of EER4 or its co-regulator by acetylating the histones of their promoter region.

**HAC4 is different from other HAC members**

From the phenotype analysis, we showed that HAC4 functions antagonistically to the other three HAC genes. The HAT domain of HAC4 is significantly different from that of the other three HACs. The vector NT blast result showed that HAC4 lacked the 267–283 fragment compared with the other three HAC proteins (Supplementary Fig. S1). Moreover, the KIX domain of CBP/p300, which mediates the interaction with CREB, p53 and numerous transcriptional activators, is composed of three \(\alpha\)-helixes. The KIX domain of HAC1, HAC5 and HAC12 contains three \(\alpha\)-helixes with linker sequence in between, while HAC4 only contains two \(\alpha\)-helixes without any linker sequence in between (Pandey et al. 2002). We speculate that these structural differences lead to the particular function of HAC4.

**Strategy to search the targets of HACs**

The pleiotropic phenotypes of the \(hac\) mutants indicate that the HACs are involved in many aspects of plant development processes. To identify functions of the HACs, one can use whole-genome ChiP-on-chip analysis to find all the target genes of the HACs. In such a method, DNA and chromatin are cross-linked, the chromatin–DNA complex is immunoprecipitated by a specific HAC antibody, the cross-linking of DNA–chromatin is

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*Fig. 6* hac mutants show pleiotropic phenotypes. (A) hac1-involved mutants show protruding gynoecia. Scale bar: 1 mm. (B) hac1-involved mutants show a short primary root. Seeds were germinated on 0.5 MS medium and grown in continuous light for 12 d. Scale bar: 2.5 cm. (C) DAPI staining of the microspore and mature pollen shows the abortion of the male gametophyte of the \(hac1hac5\) mutant. (D) hac1-involved mutants show small plant size, and dark green and wrinkly leaves. Representative plants were grown in a long-day photoperiod for 25 d. Scale bar: 2 cm.
reversed, the DNA is purified and denatured, and eventually a microarray is used to find the hybrid DNA.

Besides histones, CBP/p300 can acetylate non-histone proteins including the tumor suppressor p53, c-Myb, the nuclear receptor activator and transcription factors. In addition, p300 could self-acetylate like other acetyltransferase such as PCAF and Tip60. The acetylation of p53 is important both for the efficient recruitment of cofactors and for the activation of p53 target genes in vivo (Gu et al. 1997, Mujtaba et al. 2004). HACs might also target non-histone proteins. Yeast two-hybrid screening and immunoprecipitation followed by mass spectrometry would be a good approach to find the interaction proteins.

Materials and Methods

Plant material and growing condition

Arabidopsis thaliana [Columbia (Col-0) ecotype] plants were grown on solid MS medium supplemented with 0.9% sucrose under a 16 h light/8 h dark regime at 21°C.

The T-DNA insertional lines were obtained from the SALK collection (http://signal.salk.edu/). These lines are hac1-2 (Salk_070277), hac1-3 (Salk_080380), hac1-4 (Salk_082118), hac1-6 (Salk_122894), hac12–1 (Salk_052490), hac12–2 (Salk_071102), hac4-1(Salk_051750), hac4-2 (Salk_06923), hac4-3 (Salk_03654), hac5-1 (Salk_074472), hac5-3 (Salk_075639), hac5-4 (Salk_100792), hac5-5 (Salk_100984) and hac5-6 (Salk_085414).

Histochmical GUS staining

The promoter regions of HAC1, HAC2, HAC4 and HAC5 were inserted into the pCAMBIA1381 vector, transformed to GV3101 and then introduced into wild-type plants. Three-day-old dark-grown seedlings, and 6-day-old light-grown seedlings and inflorescences were used to check GUS expression. The seedlings were soaked in the X-Gluc staining working solution [X-Gluc working solution: 50 mM phosphate-buffered saline (PBS) pH 7.0, 10 mM EDTA, 0.1% Triton X-100, 5 mM X-Gluc] at 37°C. After 15 min, the solution was discarded, 50% glycerol was added to bleach the sample. The sample was then placed in 0.24 N HCl, 20% methanol at 57°C for 15 min. The solution was discarded, and 7% NaOH and 60% ethanol were added at room temperature for 15 min; this solution was then discarded, and the sample was treated with 40, 20 and 10% ethanol in turn, each one for 5 min; the solution was again discarded, and 5% ethanol was added for 15 min; after discarding the solution again, 25% glycerol was added for 15 min, the solution was discarded, 50% glycerol was added and a photograph was taken under the microscope.

ACC treatment in the light and in the dark

Sterilized seeds were germinated on 0.5 MS medium supplemented with 0.5 μM ACC (0.8% agar) at 4°C for 4 d, 22°C light induction for 4 h, at a light density of 8.613.5 lux, and grown in the dark at 22°C for 3 d. Photographs of hypocotyls were taken under a microscope. The average was obtained from at least 20 seedlings.

Sterilized seeds were germinated on LNM supplemented with ACC. LNM contains Millipore water and 0.7% agar, pH 5.7. The plates were put in the dark at 4°C for 3 d before transfer to 22°C, 16 h light/8 h dark for 12 d at a light density of 1,439.5 lux. The photographs of hypocotyls were taken under a microscope. The averages were from at least 20 seedlings.

Real-time PCR

The RNA extraction kit is from QIAGEN, the reverse PCR kit is from ThermoScript and the primer labeling kit is the product of Promega.

Total RNA was treated with DNase, a SUPERSCRIPT First Strand Synthesis System was used to do carry out reverse PCR in a 20 μl system. A 1 μl aliquot of the product was used to carry out the following PCR. The real-time PCR kit is the SYBR GREEN PCR Master Mix kit (ABI). The PCR program is: 95°C, 15 s; 95°C, 3 s; 60°C, 30 s; 72°C, 34 s, for 40 cycles.

Supplementary data

Supplementary data are available at PCP online.

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Disclosures

The authors have no conflicts of interest to declare.

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