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The small molecule hyperphyllin enhances leaf formation rate and mimics shoot meristem integrity defects associated with AMP1 deficiency

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SUMMARY

A chemical genetic approach identified the drug hyperphyllin, which phenocopies mutation of AMP1, a member of the M28 carboxypeptidase family with novel plant-specific functions.
This work was supported by the Austrian Science Fund (FWF; T.S.; P19935), an APART fellowship from the Austrian Academy of Sciences (ÖAW; T.S; 11300), a Ph.D. fellowship from the China Scholarship Council (CSC; S. Y.) and the Vienna Science and Technology Fund (WWTF; T.S.; LS2009-055).

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ALTERED MERISTEM PROGRAM1 (AMP1) is a member of the M28 family of carboxypeptidases with a pivotal role in plant development and stress adaptation. Its most prominent mutant defect is a unique hypertrophic shoot phenotype combining a strongly increased organ formation rate with enhanced meristem size and the formation of ectopic meristem poles. However, so far the role of AMP1 in shoot development could not be assigned to a specific molecular pathway nor is its biochemical function resolved.

In this work we evaluated the level of functional conservation between AMP1 and its human homolog HsGCPII, a tumor marker of medical interest. We show that HsGCPII cannot substitute AMP1 in planta and that an HsGCPII-specific inhibitor does not evoke amp1-specific phenotypes. We used a chemical genetic approach to identify the drug hyperphyllin (HP), which specifically mimics the shoot defects of amp1, including plastochron reduction and enlargement and multiplication of the shoot meristem. We assessed the structural requirements of HP activity and excluded that it is a novel cytokinin analog. HP-treated wild-type plants showed amp1-related tissue-specific changes of various marker genes and a significant transcriptomic overlap with the mutant. HP was ineffective in amp1 and elevated the protein levels of PHAVOLUTA, consistent with the postulated role of AMP1 in miRNA-controlled translation, further supporting an AMP1-related mode of action. Our work suggests that plant and animal members of the M28 family of proteases adopted unrelated functions. With HP we provide a novel tool to characterize the plant-specific functions of this important class of proteins.

Key words: AMP1, Arabidopsis, chemical genetics, GCPII, hyperphyllin, plastochron, shoot apical meristem
INTRODUCTION

Arabidopsis thaliana (Arabidopsis) ALTERED MERISTEM PROGRAM1 (AMP1, At3G54720, MEROPS ID: M28.007) belongs to the Zn\(^{2+}\)-dependent metalloproteases of the M28B peptidase family (Helliwell et al., 2001). Family members are found in various multi-cellular organisms and share the following protein motifs: an N-terminal trans-membrane domain, a protease-associated (PA) domain and a M28 peptidase motif followed by a transferrin receptor dimerization domain (Davis et al., 2005; Mesters et al., 2006). In strong contrast to the wealth of genetic data positioning AMP1 as a crucial component for proper plant development and hormonal responses, a coherent understanding of its biochemical function(s) is lacking.

The most prominent defect ofAMP1 loss-of-function mutants is hypertrophic activity of the shoot apical meristem (SAM). Mutant embryos form a larger SAM with supernumerary cotyledons and development of true leaf primordia starts before germination (Conway and Poethig, 1997; Vidaurre et al., 2007). During the vegetative growth phase the enlarged mutant shoot apex generates leaves at a much higher pace and with altered phyllotaxis (Chaudhury et al., 1993; Nogué et al., 2000a). Moreover, vegetative SAM enlargement and increased organ formation rate correlate and might be at least partially driven by a strong tendency to generate ectopic stem cell niches in the shoot apical meristem periphery (Huang et al., 2015). Similar SAM-related phenotypes were also observed in mutants ofAMP1 orthologs of Zea mays, Oryza sativa and Lotus japonicus, suggesting that its role in shoot development is conserved among flowering plants (Suzuki et al., 2008; Kawakatsu et al., 2009; Suzaki et al., 2013).

Additional phenotypes ofamp1 in seemingly unrelated processes were described including constitutive photomorphogenesis, ecotype dependent alterations in germination and flowering time (Chaudhury et al., 1993; Lee, 2009; Griffiths et al.,
synergistically in the embryo sac (Kong et al., 2015), suspensor proliferation in the presence of an intact embryo (Vidaurre et al., 2007), increased capacity for somatic embryogenesis (Mordhorst et al., 1998) and elevated abiotic stress resistance (Shi H et al., 2013; Shi Y et al., 2013; Yao et al., 2014). AMP1 mutant plants exhibit obvious alterations in the biosynthesis of and response to plant hormones, however, to deduce the range of phenotypes from defects in one of the classical hormonal pathways turned out to be difficult. Cytokinin (CK) biosynthesis has been shown to be upregulated in amp1 (Chin-Atkins et al., 1996; Nogué et al., 2000b; Saibo et al., 2007; Huang et al., 2015). Whereas the increased CK levels appear to be responsible for de-etiolation in the dark, increased shoot branching and enhanced tolerance against nitric oxide (Liu et al., 2013) it has been recently shown that they are rather consequence than cause of the abnormal SAM phenotypes found in the mutant (Huang et al., 2015). Depending of the subset of phenotypes analysed, several studies also reported alterations in other hormonal pathways including ethylene, gibberellin, abscisic acid and auxin (Saibo et al., 2007; Vidaurre et al., 2007; Griffiths et al., 2011; Shi H et al., 2013; Shi Y et al., 2013; Yao et al., 2014). The unique pleiotropic mutant phenotype might result from a multifunctional role of AMP1 exerting distinct functions in unrelated processes similar to human GCPII (see below), e.g. by the independent use of individual protein domains. However, this assumption is not supported by the phenotypic similarity of the relative high number of characterized amp1 alleles, where separation of individual phenotypes was not described yet. Thus, it is more likely that AMP1 either acts in a novel signaling pathway, which controls several processes analogous to known plant hormones or fulfills a basic cell biological or metabolic “house-keeping” function important for numerous regulatory pathways. A recent study supports the latter hypothesis, showing that endoplasmic reticulum (ER)-associated translation of miRNA-targeted transcripts is limited by the presence of AMP1 and its paralog LAMP1, pointing towards a general
de-regulation of miRNA-regulated pathways in the mutant (Li et al., 2013). However, further specification of AMP1 molecular function by genetic means was so far not successful. Loss of function double mutants of the cytochrome P450 genes \textit{CYP78A5/CLUH} and \textit{CYP78A7} as well as gain of function alleles of the multi-drug and toxic compound extrusion (MATE) transporter gene \textit{ZRIZI} bear phenotypic similarities to \textit{amp1}. However, their genetic interactions and levels of phenotypic overlap have not been described in detail and the biochemical functions of the corresponding gene products are unidentified (Wang et al., 2008; Burko et al., 2011).

Moreover, no attempts were described to reveal AMP1 enzymatic activity by functional comparison with characterized members of the M28B peptidase family. The closest and best-studied AMP1 homolog in animals, human glutamate carboxypeptidase II (HsGCPII; 28% amino acid sequence identity to AMP1), also known as prostate-specific membrane antigen and folate hydrolase 1, exerts different activities in a tissue-specific context. In neuronal tissues, HsGCPII cleaves the peptide N-acetyl-L-aspartyl-L-glutamate (NAAG) into N-acetyl-L-aspartate and L-glutamate (Robinson et al., 1987; Klusak et al., 2009). NAAG is the most abundant neuropeptide in the brain and is thought to act as reservoir for GCPII-dependent release of the neurotransmitter L-glutamate. In the brush border of the small intestine the same enzyme also exerts foly-
poly-\(\gamma\)-Glu carboxypeptidase activity, which is thought to mediate dietary folate uptake (Pinto et al., 1996; Navratil et al., 2014). The \textit{in vivo} requirement of these biochemical activities could not be clearly demonstrated since knock out mice of GCPII show normal development and behavior, most likely because of functional redundancy with the close paralog GCPIII (Bacich et al., 2002; Gao et al., 2015). GCPII is also expressed at lower levels in various other tissues and is strongly upregulated in prostate cancer cells and neo-vasculature of most solid-tumors. However, its physiological function in these tissues and during tumor development is not understood and additional non-proteolytic activities of GCPII are discussed (Barinka et al., 2012).
Despite this lack of knowledge, GCPII has developed into a very important pharmacological target. Small molecule inhibitors of GCPII have been reported to attenuate neuronal damage associated with glutamate excitotoxicity, a process linked to various acute and chronic disorders including stroke, Alzheimer’s dementia and Parkinson’s disease (Barinka et al., 2012). Additionally, based on its membrane localization and strong upregulation in prostate cancer and metastatic tissues, GCPII constitutes a key target for prostate cancer diagnosis and therapy (Barinka et al., 2012). Taken together, a better molecular characterization of members of this protease family is of high relevance for the plant sciences and beyond.

Here we present evidence that AMP1 and its human homolog HsGCPII are functionally divergent. To further elucidate AMP1 molecular function we undertook a chemical genetic screen and identified the small molecule hyperphyllin (HP), which specifically mimics amp1-related phenotypes, including shoot meristem enlargement, ectopic SCN formation and plastochron reduction. Consistent with the phenotypic resemblance, HP-treated wild-type plants displayed amp1-related tissue-specific changes of various marker genes and transcriptomic analysis revealed a significant overlap in changes of global gene expression. Amp1 was only marginally responsive to HP treatment in phenotypic and molecular analyses. Moreover, HP elevated the protein levels of the miRNA-regulated HDZIPIII transcription factor PHAVOLUTA (PHV) in wild type, further emphasizing an AMP1-related mechanism of action. To our knowledge HP represents the first chemical promoter of leaf formation and constitutes a novel tool to further characterize AMP1’s pivotal role in plant development.

RESULTS

No evidence for functional conservation between AMP1 and HsGCPII
To analyse the level of functional conservation between AMP1 and its best-characterized homolog human glutamate carboxypeptidase II (HsGCPII) we determined, which of the amino acid signatures shown to be required for HsGCPII substrate recognition and catalysis are conserved in the Arabidopsis protein. All of the zinc-coordinating residues as well as the catalytic glutamate residue for peptide cleavage are present in AMP1 suggesting that it exerts peptidase activity as its human counterpart (Fig. 1A; Supplemental Fig. S1A). However, of the residues defining the substrate binding pockets S1 and S1’ only 4 out of 15 (27%) amino acids are conserved in AMP1 with a specifically high variation in the S1 site indicating that substrate-specificities might differ between the two proteins. To further investigate these differences we expressed different versions of recombinant AMP1 protein to test its catalytic activity against the HsGCPII substrates N-acetyl-L-aspartyl-L-glutamate (NAAG) and folyl-poly-γ-L-glutamic acids. However, we were not able to produce sufficient amounts of AMP1 protein using either *Escherichia coli* or the insect cell expression system, which is routinely used for recombinant GCPII production (Tykvart et al., 2012). As an alternative approach to test for functional conservation between the two proteins, we expressed a MYC-tagged version of HsGCPII under control of the CaMV 35S promoter in *amp1-1*. We obtained several independent 35S::GCPII-MYC lines, which strongly expressed HsGCPII in *amp1-1*, but none of them showed phenotypic rescue of any of the known *amp1-1* growth defects (Fig. 1, B and C). We also tested the effect of the well-characterized chemical HsGCPII inhibitor 4,4’-phosphinicobis(butane-1,3-dicarboxylic acid) (PBDA) (Zhou et al., 2005) on seedling development. Germination and growth for 10 days in liquid medium containing 50 μM PBDA did not induce any visible *amp1*-like shoot defects in wild type (Fig. 1D). Treatment on solid medium containing 100 μM PBDA caused reduced leaf expansion and inhibition of primary root growth (Supplemental Fig. S1B). Again, application of neither 10 μM nor 100 μM PBDA to wild type provoked any hypertrophic shoot
phenotypes reminiscent to amp1-1 (Supplemental Fig. S1B). Taken together, we could
not find any evidence for the conservation of the biochemical functions of HsGCPII and
AMP1.

Chemical genetic identification of hyperphyllin, a small molecule that causes
SAM phenotypes reminiscent of amp1
To identify novel chemical tools to elucidate the biochemical function of AMP1 we
performed a two-tier combinatorial small molecule screen (Supplemental Fig. S2). 7800
compounds (Ion Channel Set, ChemBridge) were applied in parallel to wild-type
and amp1-1 seedlings and we screened for chemicals, which either rescue the amp1-
mutant seedling phenotype or mimic the amp1-phenotype in wild type. Hits of the first
category might include compounds with structural similarity to the product(s) of AMP1
catalytic activity, whereas hits of the second category might represent potential
inhibitors of the enzyme (Supplemental Fig. S2). Both genotypes were germinated in
liquid medium containing the chemicals and phenotypes were scored by visual
inspection 10 days after germination. Whereas there was no hit of the first category we
identified one compound that triggered amp1-specific phenotypes in wild-type plants
(Fig. 2). Since one main characteristic of amp1 is the enhanced vegetative leaf
formation rate, which leads to more than twice the number of leaves compared to wild
type at 10 days after germination (Fig. 2, A, I and M), we named the compound
hyperphyllin (HP). Wild-type seedlings treated with 30 μM HP showed a 41% increase
in leaf number compared to the solvent (DMSO) control (Fig. 2, E and M). The
enhanced leaf formation rate of HP-treated seedlings was associated with an
increased mitotic CYCLIN B1;1 (CYCB1;1)::GUS reporter expression in the shoot
meristematic area and in young leaf primordia, similar to amp1-1 (Fig. 2, B, F and J).
HP not only affected leaf formation rate, it also caused a reduced leaf blade width,
another feature of amp1-1 (Fig. 2, E and I). A third hallmark of amp1 seedlings is
increased vegetative SAM size. Under our growth conditions, *amp1*-1 SAMs were 3.4 times the size of those of wild type (Fig. 2, C, D, K, L and N). Application of HP enlarged the meristematic area in the wild-type shoot apex by a factor of 2.3 (Fig. 2, G, H and N). Treated SAM domes showed an increased height as well as lateral expansion most likely caused by higher cell numbers in the L3 layer, which can also be found in a more extreme manner in *amp1*-1. Taken together, HP triggers a spectrum of shoot phenotypes typical for *amp1*.

**Structural requirements of hyperphyllin activity**

HP (N-[4-amino-2-chlorophenyl]-2,4-dichlorobenzamide) consists of two substituted benzene rings connected by an amide bond (Fig. 3A). The leaf formation enhancing activity of HP started at a concentration of 10 μM and peaked at 50 μM with higher concentrations being less effective due to a general toxic effect (Fig. 3B). To assess the minimal structural requirements of HP activity we compared a number of analogs for their effect on leaf organ formation at a concentration of 50 μM (Fig. 3, A and C). The un-substituted benzanilide (I2) and 3-chloro-benzanilide (I1) were inactive, whereas 2-chloro-benzanilide (A3) exerted weak activity (Fig. 3c). An additional fluor in ortho-position of ring 2 (2-chloro-6-fluoro-N-phenylbenzamide, A2) resulted in a more active compound, which already showed a measurable effect at 1 μM concentration and a comparable peak activity to HP at 50 μM (Fig. 3, B and C). Compound A1 (4-chloro-N-[2,6-difluorophenyl]benzamide) showed the highest potency in this analysis, which reached already 50% of its maximal activity at 1 μM concentration (Fig. 3, B and C). To better visualize the structure/activity relationship of the tested analogs we performed a structural clustering analysis using the ChemMine software (Backman et al., 2011). Based on this algorithm, HP is most closely related to A1 and the two other active compounds A2 and A3 build a second distinct branch (Fig. 3C). All four active compounds have halogen substituents in ring 2 in ortho- and/or para-position. If ring 2
is either un-substituted (I2), halogenated in meta (I1) or the halogen in ortho is exchanged to an amino group (I3), activity is lost. In contrast, there is considerable structural freedom at ring 1.

**Activities of shoot apical meristem markers are highly similar in amp1 and hyperphyllin-treated wild-type plants**

To test whether the observed morphological similarities between HP-treated wild-type plants and amp1 also correlate with changes in underlying molecular processes we analyzed the expression of marker genes known to be specifically altered in the mutant. The SAM boundary marker KLUH/CYP78A5, which controls leaf formation rate, is massively upregulated in amp1-1 (Zondlo and Irish, 1999; Helliwell et al., 2001; Wang et al., 2008). To assess whether KLUH/CYP78A5 expression is also affected by HP we used the pKLU::GUS reporter (Anastasiou et al., 2007). In amp1-1, pKLU::GUS activity was not only found in a much broader domain depicting the enlarged SAM area, the staining also appeared to be more intense compared to the mock-treated wild-type control (Fig. 4A). In HP-treated wild-type plants the expression of the reporter was also clearly expanded and stronger compared to the mock control, however, not to the same extent as in amp1-1 (Fig. 4A). Another marker with an altered expression pattern in amp1 is the organizing center (OC) defining transcription factor WUSCHEL (WUS) (Huang et al., 2015). The zone of WUS::GUS activity was substantially broadened in both amp1-1 and in drug-treated wild-type SAMs compared to the mock-treated wild-type control (Fig. 4B). Moreover, the previously reported amp1-specific formation of ectopic OCs in the SAM periphery (Huang et al., 2015) was phenocopied in wild type by HP-treatment (Fig. 4, C and D). Under the growth conditions used, around 20% of amp1 plants showed more than one WUS::GUS expression domain in the primary SAM. Notably, this characteristic behavior was also observed in 7% of HP-treated plants, but absent in the mock-control (Fig. 4C). The expanded and partially
ectopic expression of the OC marker WUS in amp1 and HP-exposed wild type was
accompanied with the formation of enlarged and partially fragmented CLAVATA3
(CLV3)-expressing stem cell pools in the shoot apex (Fig. 4E). Furthermore, HP
triggered ectopic CLV3::GUS activity in vascular tissues of petioles, hypocotyl and
roots, another specific characteristic of amp1 (Fig. 4E).

Hyperphyllin mediates cytokinin-independent effects

Plants mutated in amp1 show elevated cytokinin biosynthesis, which results in
enhanced cytokinin-responsive reporter activity particularly in vascular-associated
tissues (Chaudhury et al., 1993; Nogué et al., 2000b; Saibo et al., 2007; Huang et al.,
2015). However, this defect in cytokinin biosynthesis appears to be rather a
consequence than a cause of the shoot phenotypes of amp1 (Huang et al., 2015). To
test whether HP affects cytokinin homeostasis we used the cytokinin responsive
ARABIDOPSIS RESPONSE REGULATOR5 (ARR5)::GUS reporter as a read out (Fig.
5A). Almost identical to amp1, we observed augmented activity of ARR5::GUS in the
SAM, hypocotyl and petioles of HP-treated seedlings. This raised the question whether
HP exerts cytokinin activity on its own. This scenario seemed rather unlikely, since HP
has no obvious structural similarity to any of the known cytokinins. To further exclude
this possibility, we compared the effect of exogenously applied trans-zeatin and HP on
leaf formation in a dose response experiment. In contrast to HP, we did not observe
any leaf number promoting effect of trans-zeatin under our growth conditions in a
concentration range from 1 μM to 75 μM (Fig. 5, B and C). Correspondingly,
transcriptomic analysis of HP-treated plants (see below) revealed only a marginal
overlap with the published transcriptomic responses of cytokinin-treated plants strongly
supporting a model, in which HP does not directly exert cytokinin activity.
Hyperphyllin-treated wild-type and amp1 plants show significantly overlapping transcriptomic alterations

If the observed amp1-mimicking effect of HP is based on molecular interference with the AMP1 pathway one would expect overlapping transcriptomic aberrations in the mutant and in wild type after compound treatment. To test this assumption, we performed a genome-wide transcript analysis using the Arabidopsis ATH1 Genome Array (Affymetrix). Wild-type seedlings were germinated and grown for 10 days in liquid medium in the presence of either DMSO or 30 μM HP, whereas amp1-13 seedlings were grown under the same conditions in medium containing only DMSO. HP-treatment of wild type altered the expression of 1526 genes (978 induced/548 repressed) whereas 953 genes (645 induced/308 repressed) were misexpressed in amp1-13 (Fig. 6A). Remarkably, between these two groups there was an overlap of 608 genes (40% of the HP altered genes and 64% of amp1-13 altered genes). Moreover, only 3 out of these 608 genes showed opposite misexpression between the two samples.

To assess whether the transcriptional changes in the two samples show specific hormonal signatures, we determined the frequency of marker genes for six different hormones (Nemhauser et al., 2006). Surprisingly, in both samples less than 2% of cytokinin responsive marker genes were present, showing a similar low overlap as with ethylene and brassinosteroid-dependent marker genes (Fig. 6B). In contrast and in accordance with recent reports (Shi H et al., 2013; Shi Y et al., 2013), there was a relatively high frequency of misregulation of ABA-specific marker genes in amp1-13, which we also found in HP-treated wild type. Notably, the strongest overlap was observed with genes specifically induced by methyl jasmonate (Fig. 6B; 14% present in the HP-treated wild-type sample/12% present in the amp1-13 sample).

To identify specific biological and molecular processes commonly affected, we performed an enrichment analysis of Gene Ontology (GO) terms with the group of 608
overlapping genes using the Revigo software (Supek et al., 2011). We found a significant enrichment of genes associated with stress responses triggered by xenobiotic compounds (organic substance, chemical stimulus) and biotic interactions (defense, chitin, wounding, jasmonic acid, salicylic acid, immune response) (Supplemental Fig. S3). More specific processes like lipid localization and transport, photosynthesis, cell wall organization and trehalose metabolism also emerged as significantly affected in this analysis.

Hyperphyllin responses are substantially alleviated in the amp1 mutant background

To further analyze the specificity of HP for the AMP1-regulatory pathway, we tested the effect of HP on the leaf formation rate of amp1-1 and amp1-13. Both alleles did not show an HP-induced rise of leaf number (Fig. 7A). In contrast, clavata1-1 (clv1-1) and clavata3-2 (clv3-2) displayed a significantly elevated leaf count in the presence of the drug indicating that HP is not generally ineffective in increased meristem size mutants (Fig. 7A). We also tested the effect of HP on a mutant in the AMP1 paralog LAMP1. In this assay lamp1-2 showed an already slightly higher leaf number under control conditions compared to WT but reached similar organ numbers in the presence of HP (Fig. 7A). Thus, whereas amp1 is insensitive to the promoting effect of HP on leaf number lamp1 is only barely resistant. We also did not observe a gain in amp1-1 SAM size after HP treatment but rather a slight but insignificant negative impact similar to the leaf number analysis in the mutant (Fig. 7B, C).

Next we monitored how AMP1 specific marker genes, whose expression responded to HP in wild type, react in amp1-1 in the presence of the drug. The enhanced and ectopic activities of pKLU::GUS, ARR5::GUS and CLV3::GUS in amp1-1 were subtly further intensified by HP application, however the level of change was far weaker than
that observed in wild type (Fig. 7D). The increased WUS::GUS activity in *amp1-1* primary stem cell pools and the OC amplification rate were unaffected by HP (Fig. 7D).

To further dissect to which extent HP-induced molecular responses are dependent on AMP1 function we repeated the transcriptomic analysis expanded for a set of HP-treated *amp1* samples using the novel Ara Gene 1.1 ST Array Strips (Supplemental Tables S3 and S4). In this experiment we again detected a substantial overlap between the group of HP-regulated genes in wild type (142/496: 29%) with the set of misregulated genes in *amp1-13* (142/302: 47%) (Fig. 7E). In comparison to ATH1 chip analysis (Fig. 6A) the overall number of genes with altered expression in the different samples were considerably smaller (approximately only 30%) consistent with previous observed differences in signal strength between the two microarray platforms (Kakei and Shimada, 2015). Notably, in contrast to wild type, *amp1-13* reacted only marginally to HP application with only 60 genes affected in total. Of these, 27% (16 genes) were also present in the group of genes regulated by HP in wild type but not changed in *amp1-13* under control conditions. Thus, in the absence of AMP1, HP provokes only very limited transcriptional responses concordant with the remarkably mild phenotypic drug effects on the mutant.

Hyperphyllin elevates protein levels of a miRNA regulated member of the HDZIPIII family

Since recently it has been shown that protein levels of miRNA-regulated genes, like transcription factors of the HD-ZIPIII family, are upregulated in *amp1* due to enhanced translation by ER-localized ribosomes (Li et al., 2013) we also examined the effect of 30 μM HP or 30 μM A1 on PHV-MYC protein levels in a 35S::PHV-MYC line. Both compounds increased PHV-MYC levels as found in untreated *amp1-1* plants supporting an AMP1-dependent mode of function of these chemicals (Fig. 8A). This effect was also observed for YFP-tagged PHV in a 35S::PHV-YFP line although to a
lesser extent (Fig. 8A). The transcript levels of each of the transgenes were only slightly different between control and HP-treated samples suggesting that the observed changes at the protein level derived at least partially from a posttranscriptional effect of the compound. (Fig. 8B).

Hyperphyllin shows efficient uptake characteristics and high stability in planta

Cellular uptake and intracellular stability are important parameters affecting compound activity. We followed the accumulation of HP in seedlings grown in liquid medium over time. HP was rapidly and efficiently incorporated in wild type and intracellular concentrations reached a plateau after 24 hours (Supplemental Fig. S4). The in planta levels exceeded those of the medium several-fold. HP appeared to be relatively stable and not metabolized once taken up by the plant since concentrations stayed close to peak levels even after 50 hours of incubation. Finally, we could not find any differences between wild type and amp1-13 in this assay, suggesting that the unresponsiveness of amp1 to HP is not due to altered uptake or fate of the compound in the mutant.

DISCUSSION

Based on our protein sequence comparison, complementation approach and pharmacological analysis we conclude that AMP1 might exhibit peptidic hydrolase activity as its human homolog HsGCPII, but that the substrate specificity or other non-enzymatic key functions are most likely different between both proteins. Although we cannot fully exclude that the unsuccessful complementation of amp1 by HsGCPII was due to incorrect processing or subcellular targeting of the human protein in a plant cell environment, this does not seem to be a general issue (Hosein et al., 2010). Functional divergence between AMP1 and HsGCPII is not too surprising: 1) Both proteins show a low overall sequence identity of only 28%. 2) To our knowledge, no physiological role
was ascribed to NAAG (spaglumic acid) in plants and NAAG treatment of wild-type and
amp1 plants did not have any obvious phenotypic effects (Helliwell et al., 2001). 3) Plants synthesize folates de-novo and the majority of the in planta folate pool is poly-glutamylated. A family of γ-glutamyl-hydrolases was characterized in tomato (Solanum lycopersicum) and Arabidopsis, and they have been shown to regulate cellular folate content and folate sequestration in the vacuole (Orsomando et al., 2005). Except for lower seed set, no obvious phenotypes were described in gain- and loss of function alleles of corresponding genes, indicating that general alterations in the homeostasis of folate poly-glutamates are not directly linked to altered shoot meristem activity (Akhtar et al., 2010). However, this does not exclude that AMP1 might have an ER-specific role in folate-deglutamylation. Nevertheless, our data support a model, in which plant and animal GCPIIs adopted different functions during evolution and that the plant-specific biochemical roles of this enzyme class have yet to be identified.

HP treatment of wild-type plants imitates the amp1-specific spectrum of phenotypes at the morphological, cellular and molecular level. Based on this unique combination of phenotypes and the observed resistance of amp1 to the chemical, we assume that HP either acts directly in the AMP1 regulatory pathway or affects a parallel pathway that converges on a common molecular target. Notably, the strength of HP-induced phenotypes, particularly on SAM size, leaf formation rate and frequency of ectopic stem cell pool formation, is significantly weaker compared to amp1. However, this might be caused by the post-embryonic application of the chemical. AMP1 mutant plants show massive SAM enlargement and presence of true leaf primordia in the mature embryo giving them a head-start in developing SAM defects in the seedling stage (Conway and Poethig, 1997; Vidaurre et al., 2007). Long-term treatment of mother plants during flower and silique development could be used to verify this assumption. We further found that HP and its analogs only induce obvious amp1-related, mainly shoot-specific phenotypes when applied in liquid growth conditions,
where shoot tissues get in direct contact with the chemical. Most likely, HP has to be present in the shoot to induce the respective phenotypes and is potentially not sufficiently transported from root to shoot tissues. This is supported by the absence of weakly acidic groups in the hitherto characterized active HP analogs, which have been reported to facilitate phloem mobility to allow systemic transport in the plant (Xuan et al., 2013). Testing alternative application methods combined with future studies of transport characteristics of known and novel HP derivatives might help to overcome these constraints.

In this context it will be also important to further resolve the functional relationship between HP, AMP1 and its paralog LAMP1. Genetic analysis revealed that AMP1 and LAMP1 have partially redundant functions in shoot development and the miRNA-dependent control of translation rate (Li et al., 2013; Huang et al., 2015), with AMP1 playing a dominant role most likely due to its spatially and temporally broader expression pattern compared to LAMP1. At the morphological level and also in respect to global transcriptional responses amp1 was almost insensitive against HP implicating that LAMP1 function is not overly compromised by the compound. Surprisingly, we did not observe any obvious increased susceptibility of lamp1 to the organ promoting properties of the drug. Whether this ostensibly controversial response also results from the above mentioned restricted postembryonic application or tissue-specific functional limitations of the drug compared to constitutive elimination of AMP1 function by mutation can not be answered at the moment.

We found a remarkable overlap in transcriptomic changes between HP-treated wild-type samples and amp1. Although increased cytokinin levels have been postulated as a hallmark of amp1, we only found a minor fraction of cytokinin marker genes to be misregulated in amp1 and the HP-treated wild-type sample. This finding is consistent with amp1-related microarray data from previous studies using different developmental stages and mutant alleles. In amp1 developing seeds as well as 14-day-old plants the
fraction of cytokinin-regulated factors in the pool of misexpressed genes was only marginal (Griffiths et al., 2011; Shi Y et al., 2013). This is also in line with recent evidence that the majority of shoot meristem defects in amp1 are also present in a cytokinin-insensitive background (Huang et al., 2015). One explanation for this discrepancy regarding the elevated cytokinin levels detected in the mutant could be that increased cytokinin abundance in amp1 is restricted to specific tissues. In support of this assumption the ARR5::GUS reporter showed upregulation mainly in vascular-associated tissues of amp1. This might disguise global transcriptional responses at the whole plant level. Alternatively, general cytokinin responses might be suppressed in an AMP1-deficient background. Importantly, HP-induced transcriptional responses show a similarly low impact on the cytokinin pathway concordant with our hypothesis that the compound affects plant development in an AMP1-related manner.

Our transcriptomic analysis revealed that HP application and AMP1 deficiency alter the expression of a substantial overlapping set of ABA-responsive genes. Although these changes are consistent with recent studies where amp1 displayed elevated ABA responses and was more tolerant to ABA-related abiotic stresses (Shi H et al., 2013; Shi Y et al., 2013; Yao et al., 2014) we do not yet understand the relevance of these alterations on the observed SAM phenotypes and their meaning in terms of the potential molecular functions of AMP1 and HP. Nevertheless, it will be interesting to test whether HP treatment confers tolerance to abiotic stresses.

Another outcome of our genome wide expression comparison is the obvious coinciding effects of missing AMP1 function and HP treatment on lipid-related processes including alterations in jasmonate responses. Further studies have to be done on the physiological cause and functional relevance of these transcriptional changes to clarify, whether this information can be used to better position AMP1 and/or HP activity at the molecular level.
Thinking about HP’s mode of action, one plausible scenario is that HP directly targets the enzymatic activity of AMP1, acting as a competitive or non-competitive inhibitor. However, so far we were unable to produce evidence for physical interaction between HP and AMP1, mainly because of the difficulties to generate recombinant AMP1 in sufficient quantity and quality to perform binding studies. HP does not contain residues found in known metalloprotease inhibitors such as zinc-chelating groups resistant to hydrolysis, including hydroxamate, sulphonate phosphonate, phosphinate, phosphoramidate or urea (Zhou et al., 2005; Pavlicek et al., 2014). Based on its small size, HP might rather bind to a subdomain of the substrate-binding pocket of AMP1 similar like the N-phenylbenzamide GW9662, which inhibits the binding of fatty acid ligands to peroxisome proliferator-activated receptor gamma (Chandra et al., 2008). Thus, a future key goal will be the development of experimental strategies to assess the level of physical HP-AMP1 interaction. Testing different versions of closely related orthologs of AMP1 in different expression systems might allow the production of sufficient recombinant protein for binding studies, protease activity assays and for gathering structural information about AMP1. This step will be also critical to further compare the functional relationship between AMP1, LAMP1 and their animal homologs in the M28 family of metalloproteases such as testing whether the recently identified arene-binding site of GCPII is conserved in AMP1 and if yes, whether it plays a role in HP-AMP1 interaction (Zhang et al., 2010). In case it turns out that HP directly targets AMP1, it will be a valuable tool to further establish the biochemical function of the protein. It could be also used to further dissect the diverse roles of AMP1 in plant development by manipulating AMP1 activity in a spatially and temporally controlled manner. Moreover, it will be interesting to test to which extent HP is able to modulate other AMP1-dependent processes including egg cell fate determination, somatic embryogenesis or ABA-mediated abiotic stress resistance (Mordhorst et al., 1998; Shi H et al., 2013; Shi Y et al., 2013; Kong et al., 2015).
In case HP does not act on AMP1 directly, it may serve as a useful agent in search for additional components of the AMP1-regulatory pathway. To our knowledge, HP is the first small molecule compound, which accelerates leaf formation rate, a process not well understood at the molecular level (Lee and Jackson, 2009). It should thus also help to interrogate and specify the communication events between established organ primordia and incipient leaf anlagen regulating plastochron length.

MATERIALS AND METHODS

Plant material and growth conditions

*Arabidopsis thaliana* L. (Heynh) ecotypes Columbia (Col-0) and Landsberg erecta (Ler) were used in this study. *Amp1-1* (Col-0; N8324), *amp1-13* (Col-0; N522988), *pt* (Ler; N235), *lamp1-2* (Col-0; SM_3.22750), *clv1-1* (Ler; N45) and *clv3-2* (Ler; N8066) were ordered from the European Arabidopsis Stock Centre (NASC). Origin of transgenic lines: *CYCB1;1::GUS* (in Col-0) was provided by John Celenza (DiDonato et al., 2004), *CLV3::GUS* and *WUS::GUS* (in Ler) were received from Thomas Laux (Gross-Hardt et al., 2002), *pKLU::GUS* (in Ler) was provided by Michael Lenhard (Anastasiou et al., 2007), *ARR5::GUS* (in Col-0) was provided by Joe Kieber (D'Agostino et al., 2000). Combinations of mutants and reporter lines were obtained by crossing individual lines and genotypes were verified phenotypically and by PCR genotyping. Homozygosity of reporters was determined by unanimous GUS signal presence (n≥30).

Seeds were surface sterilized with 70% ethanol + 0.05% sodium dodecyl sulfate (SDS) for 3 min, rinsed with 96% ethanol for 1 min and subsequently dried in laminar flow hood. For the small molecule screen, GUS-reporter assays, the microarray experiment, leaf formation rate and SAM analyses, sterile seeds were germinated and grown in 96-
well plates (Nunclon Delta Surface, Thermo Fisher Scientific/Nunc 163320) (approx. 5 seeds per well) in 100 μl liquid half-strength Murashige and Skoog medium (Duchefa) containing 1% sucrose. After stratification at 4°C in darkness for 2 days, seeds were placed in a growth chamber (BrightBoy® GroBank, Model BB-XXL, CLF Plant Climatics) and grown at 26°C grown under long day conditions (16 h, 80 μM m⁻² s⁻¹ cool white light/8h dark). For the phenotypic analysis of 35S::GCPII:MYC lines, plants were grown on soil in a growth chamber under long day conditions (16 h 120 μM m⁻² s⁻¹ cool white light/8h dark at 18°C to 22°C). Trans-zeatin and PBDA were ordered from Sigma.

**Gene constructs and transformation**

PCR was performed with proofreading thermostable polymerase, and all clones were confirmed by sequencing. To create 35S::GCPII-MYC the human GCPII ORF was amplified by PCR with primers PSMAF-EcoRV and PSMAR-BamHI and subcloned into pGEM-T Easy. The fragment was excised via EcoRV and BamHI and ligated into pGWR8 (Rozhon et al., 2010) resulting in p35S::GCPII. A 6xMYC-tag (from pGWR8-MYC) was inserted into the NotI site of p35S::GCPII to create p35S::GCPII-MYC. To create 35S::PHV-MYC and 35S::PHV-YFP, the PHV ORF was amplified from cDNA (Col-0 seedlings) by PCR with primers PHV ORF F (EcoRV) and PHV ORF R (NotI). The fragment was subcloned into pGEM-T Easy. Subsequently, the PHV ORF was transferred via EcoRV and NotI into the pGWR8 backbone to generate p35S::PHV. The YFP ORF (from pGWR8-YFP) and a 6xMYC-tag (from pGWR8-MYC) sequence were subcloned into the NotI site of p35S:PHV to create p35S::PHV-YFP and p35S::PHV-MYC, respectively.

Using the floral dip method, amp1-1⁻/⁻ plants were transformed with p35S::GCPII:MYC and amp1-1⁻/+ plants were transformed with p35S::PHV:YFP and p35S::PHV:MYC,
respectively (Clough and Bent, 1998). At least 10 independent transformants were generated for each line and resulting T2 lines were confirmed for single-transgene insertion sites based on the 3:1 segregation of the selection marker and propagated for further analysis. For 35S::PHV::YFP and 35S::PHV::MYC segregating wild-type and amp1-1 plants were isolated, which were isogenic and homozygous for the transgene.

Small molecule screen

For the primary screen, 7800 chemicals of the Ion Channel Set (Chembridge) were tested under the growth conditions described above at a final concentration of 50 μM in DMSO [1% (v/v)]. 5 seeds per well were germinated and grown in the presence of the compounds until day 10, when visual inspection of leaf number and SAM size was done with a stereomicroscope (Olympus SZX10). HP and structural analogs were reordered from the following sources: HP (5839245), A1 (7679986), A2 (5578458) and I3 (6955571) were purchased from Chembridge. A3 (S571202), I1 (S883042) and I2 (442470) were ordered from Sigma. To correlate structural features of compounds with their activities the ChemMine clustering and data mining web tools were used (http://chemmine.ucr.edu/).

Leaf number analysis

The number of visible leaves was recorded after examination of the shoot apex area under the stereomicroscope (2x magnification) 10 days after germination. In each experiment, the mean number of emerged leaves from at least 10 plants was calculated.

GUS-staining

Plants were submerged in GUS staining buffer (Jefferson et al., 1987) containing 1 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (Duchefa), 100 mM sodium phosphate
(pH 7.0), 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide, 10 mM EDTA, and 0.1% (v/v) Triton X-100. Seedlings were incubated at 37°C for individual periods of time depending on the reporter strength. After staining, the samples were dehydrated in 70% ethanol. Seedlings were analysed with a stereomicroscope (Olympus SZX10) equipped with a digital camera (Olympus DP26).

**Histology**

For histological analysis, 10 day-old seedlings were fixed overnight at 4°C in FAA [10% (v/v) formaldehyde, 5% (v/v) acetic acid and 50% (v/v) ethanol]. After fixation, samples were dehydrated in an ethanol series and embedded in Technovit 7100 (Heraus Kulzer), according to the manufacturer's instructions, as described (De Smet et al., 2004). A series of 5-7 µm thick longitudinal sections were made with a rotary microtome (Reichert-Jung). Sections were transferred to microscopic slides (Marienfeld) stained with ruthenium red (Sigma) and photographed with a digital camera (PM-20) mounted on an Axiophot microscope (Zeiss).

**Scanning electron microscopy**

Seedlings were incubated in fixative (50% ethanol, 10% acetic acid, 5% formaldehyde) overnight at 4°C and then dehydrated through a graded ethanol series up to 98% ethanol and supercritical point dried using an EM CPD300 (Leica). Dried seedlings were dissected and mounted on conductive adhesive tabs (Plano) under a stereomicroscope (SZX10, Olympus). Samples were subsequently examined using a T-3000 table top SEM (Hitachi).

**Transcriptomic analysis using Arabidopsis ATH1 genome arrays**

The experiment was conducted with 10-day-old seedlings of the indicated genotypes incubated with either DMSO [1% (v/v)] or 30 μM HP under growth conditions described
Total RNA was extracted from three biological replicates for each treatment using the RNeasy Kit (Qiagen). RNA quality control, cDNA synthesis, labeling and hybridization on a GeneChip Arabidopsis ATH1 genome array (Affymetrix) were conducted at the Nottingham Arabidopsis Stock Centre according to standard Affymetrix protocols. The raw microarray data were preprocessed and normalized using the RMA (Robust Multiarray Average) method (Irizarry et al., 2003); data statistical significance was assessed by using the moderated t-test (Smyth, 2004); p-value was adjusted with FDR (Benjamini and Hochberg false discovery rate ≤0.05) (Benjamini and Hochberg, 1995). Genes with adjusted p-values less than 0.05 were filtered with regard to their differential expression (two-fold change, 95% confidence). The gene ontology terms analysis was done using AgriGO and ReviGO web-based tools (Supek et al., 2011). The microarray data were deposited in NCBI’s Gene Expression Omnibus.

Transcriptomic analysis using Arabidopsis Gene 1.1 ST Array Strips

The experiment was conducted with 10-day-old seedlings of the indicated genotypes incubated with either DMSO [1% (v/v)] or 30 μM HP under growth conditions described above. Total RNA was extracted from three biological replicates for each treatment using the E.Z.N.A. Plant RNA Kit (OMEGA Bio-tek). Sample concentration and purity of RNA was determined by spectrophotometry and integrity was confirmed using an Agilent 2100 Bioanalyzer with the RNA 6000 Nano Kit (Agilent Technologies). Whole-genome transcriptome analysis was conducted by hybridizing twelve biological samples of total RNA per treatment to Affymetrix Arabidopsis Gene 1.1 ST Array Strips (Affymetrix). All steps were conducted at the Nottingham Arabidopsis Stock Centre. Gene expression data were analyzed using Partek Genomics Suite 6.6 software (Partek Incorporated). The raw CEL files were normalized using the RMA background correction with quantile normalization, log base 2 transformation and mean probe-set
summarization with adjustment for GC content. Differentially expressed genes (DEG) were identified by a two-way ANOVA. DEG were considered significant if p-value was ≤ 0.05 at a fold-change (FC) of >2 or <-2. The microarray data were deposited in NCBI's Gene Expression Omnibus.

Quantitative real-time RT-PCR

For qPCR about 50 mg of seedlings was collected and shock frozen in liquid nitrogen. After total RNA extraction with the E.N.Z.A. Plant RNA Mini Kit (OMEGA Bio-tek), DNase I (Thermo Scientific) treatment, and cDNA synthesis with RevertAid first-strand cDNA synthesis kit (Thermo Scientific), qPCR was performed with a Eppendorf realplex2 Mastercycler (Eppendorf) using SensiFAST SYBR Lo-ROX Mix,2x (Bioline) and specific primers for the mRNAs of interest. Data were normalized to UBC (AT5G25760) and measured in at least three technical replicates.

Protein preparation and immunoblotting

Plant material (50 mg) was flash-frozen in liquid nitrogen and homogenized with a Retsch mill. 200 μl extraction buffer (62.5 mM TRIS pH 6.8, 125 mM DTT, 2.5% SDS, 12.5% glycerol, 0.01% bromophenol blue) was added and samples were incubated at 95°C for 2 min. The samples were centrifuged at 14000 g for 5 min and 10 μl of the supernatants separated by SDS-PAGE (10% gel) and semidry-blotted onto a polyvinylidene difluoride membrane (Millipore) The membrane was blocked with blocking buffer (5% skim milk powder dissolved in 0.05% Tween 20, 150 mM NaCl and 10 mM TRIS/HCl pH 8.0). For PHV-YFP detection the membrane was probed with a mouse anti-GFP-horseradish peroxidase antibody (1:5000; Miltenyi Biotec) and signals were detected using ECL Select Detection Reagent (GE Healthcare). For PHV-MYC detection membranes were probed with a mouse anti-c-Myc antibody (1:5000; Santa Cruz Biotechnology). Alkaline phosphatase-conjugated goat anti-mouse IgG (Sigma)
diluted 1:5000 with blocking buffer was employed as secondary antibody. For detection
the CDP-Star detection reagent (GE Healthcare) was used.

**Hyperphyllin uptake assay**

Whole seedlings were grown for 7 days on agar medium (half-strength Murashige and
Skoog) and transferred to liquid medium (approx. 50-70 mg plant material/ml half-
strength Murashige and Skoog medium). At certain time points HP was added to a final
concentration 50 µM. After the treatment, plants were frozen in liquid nitrogen. For
analysis, the plant material was ground to a fine powder and 1 ml of 40% acetonitrile
(ACN) was added to each sample. The mixtures were spiked with 100 µl of a 100 µM
2-chloro-benzanilide (A3) stock solution as internal standard. Extraction was performed
in a thermomixer set to 60°C and 800 rpm for 1 h. The extracts were centrifuged for 5
min at 15,000 g. The clear supernatants were diluted with 2 ml 20 mM TRIS/HCl pH
8.0 and loaded onto C8 100 mg solid phase extraction columns (Macherey-Nagel)
conditioned with 1 ml 100% ACN and subsequently with 1 ml 20% ACN. The columns
were washed with 1 ml 30% ACN. Subsequently, elution was performed with 1 ml 60%
ACN and the eluates were directly used for HPLC. A LC-10 system (Shimadzu)
equipped with a Symmetry 3.5 µm C18 100 x 4.6 mm column (Waters, Milford, MA)
was used for HPLC analysis. The injection volume was set to 100 µl. Elution began
with an isocratic flow of 1 ml/min of 67.7% solvent A (20 mM acetic acid set with NaOH
to pH 4.8 in 15% ACN) and 32.3% solvent B (80% ACN) for 15 min. The concentration
of solvent B was then raised linearly to 100% in 0.5 min and kept isocratic for another 2
min prior to reducing it to 32.3% within 0.5 min. Finally, the column was equilibrated
with 67.7% solvent A and 32.3% solvent B for 7 min before injection of the next
sample. The absorbance was recorded at 245 nm for quantification and 260 nm for
purity check. The obtained chromatograms were analysed with the Clarity software
(DataApex).
SUPPLEMENTAL DATA

Supplemental Figure S1. Protein sequence alignment of HsGCPII with AtAMP1 and analysis of PBDA treatment of solid-medium grown Arabidopsis plants.

Supplemental Figure S2. Strategy and setup of an AMP1-specific chemical genetic screen in Arabidopsis.

Supplemental Figure S3. Enrichment analysis of gene ontology terms of genes misregulated both in Arabidopsis HP-treated wild type and amp1-13.

Supplemental Figure S4. Enrichment analysis of gene ontology terms of HP-regulated genes in amp1-13.

Supplemental Figure S5. HP shows efficient uptake characteristics and high stability in planta.

Supplemental Table S1. Differentially expressed genes identified in the transcriptomic analysis using Arabidopsis ATH1 genome arrays.

Supplemental Table S2. REVIGO gene ontology enrichment analysis of genes misregulated both in HP-treated wild type and amp1-13.

Supplemental Table S3. Differentially expressed genes identified in the transcriptomic analysis using Arabidopsis Gene 1.1 ST Array Strips.

Supplemental Table S4. List of primers used in this study.

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AUTHOR CONTRIBUTIONS
T.S. and P.M. designed the research; O.P., S.Y., D.P., W.R., K.Z., and M.C.U. performed the research; O.P., S.Y., D.P., W.R., K.Z., M.C.U., S.M. B.P. and T.S. analyzed the data; T.S. wrote the article.

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FIGURE LEGENDS

Figure 1. Analysis of the degree of functional conservation between Arabidopsis AMP1 and human GCPII. A, List of amino acid (aa) residues defining the indicated functional features in HsGCPII (Mesters et al., 2006) and their corresponding aa residues in AMP1 based on a protein sequence alignment shown in Supplemental Fig. S1. Divergent aa residues are indicated in green. B, Shoot phenotypes of Arabidopsis wild type, 35S::GCPII-MYC amp1-1 line 2 and amp1-1 at 14 days after germination (DAG). C, Immunoblotting of protein extracts of 2-week-old seedlings of the indicated 35S::GCPII-MYC amp1-1 lines using α-MYC antibody for GCPII-MYC detection. Upper panel: autoradiogram, lower panel: Ponceau S staining as a loading control. D, Shoot
phenotypes of Arabidopsis seedlings of the indicated genotypes grown for 10 days in liquid medium containing either only the solvent DMSO or 50 μM PBDA, WT, wild type; pt, the amp1 allele primordia timing. Bars: (B) 5 mm; (D) 1 mm.

Figure 2. Hyperphyllin (HP) treatment phenocopies amp1-associated shoot defects. A, E and I, Comparison of Arabidopsis wild-type (A and E) and amp1-1 (I) seedlings grown for 10 days in liquid medium containing either only the solvent DMSO (A and I) or 30 μM hyperphyllin (HP, E). B, F and J, Comparison of CYCB1;1::GUS activity in Arabidopsis wild-type (B and F) and amp1-1 (J) seedlings grown for 10 days in liquid medium containing either only the solvent DMSO (B and J) or 30 μM HP (F). C, G and K, Median longitudinal SAM sections of Arabidopsis wild-type (C and G) and amp1-1 (K) seedlings grown for 10 days in liquid medium containing either only the solvent DMSO (C and K) or 30 μM HP (G). Scanning electron micrographs of SAM areas of Arabidopsis wild-type (D and H) and amp1-1 (L) seedlings grown for 10 days in liquid medium containing either only the solvent DMSO (D and L) or 30 μM HP (H). M, Quantification of leaf number in mock-treated wild type (WT+DMSO), wild type treated with 30 μM HP (WT+HP) and mock-treated amp1-1 (amp1-1+DMSO) at 10 DAG grown in liquid medium. Values are means ± SE (n ≥ 30). N, Quantification of SAM size from median longitudinal sections of mock-treated wild type (WT+DMSO), wild type treated with 30 μM HP (WT+HP) and mock-treated amp1-1 (amp1-1+DMSO) at 10 DAG grown in liquid medium. Values are means ± SE (n ≥ 3). Bars: (A, E and I) 2 mm; (B, F and J) 1 mm; (C, D, G, H, K and L) 50 μm.

Figure 3. Structure-activity relationship of hyperphyllin (HP) and its analogs. A, Chemical structures of HP and its active (A1, A2, A3) and inactive (I1, I2, I3) analogs. B, Effects of HP and analogs A1 and A2 on leaf number in Arabidopsis wild-type seedlings grown for 10 days in liquid medium containing the indicated concentrations
of the compounds. Values are means ± SE (n ≥ 10). C, Correlation of structure-based hierarchical clustering of HP and its analogs with their respective effects on the number of leaves formed at a concentration of 50 µM. Values are means ± SE (n ≥ 30). Hierarchical clustering was generated by the ChemMine clustering tool (see materials and methods).

**Figure 4.** Shoot apical meristem (SAM) marker activities in Arabidopsis hyperphyllin (HP)-treated wild-type plants are highly reminiscent to those of *amp1*. Comparison of SAM marker activities in Arabidopsis mock-treated wild type (WT+DMSO), wild type treated with 30 µM HP (WT+HP) and mock-treated *amp1-1* (*amp1*DMSO) at 10 DAG grown in liquid medium. A, pKLU::GUS activity. B, WUS::GUS activity. C, Percentage of plants showing ectopic WUS expression foci in the SAM (n ≥ 100). D, 30 µM HP-treated wild-type and mock-treated *amp1-1* seedling with ectopic WUS expression foci in the SAM. E, CLV3::GUS activity in the SAM (upper panel), in the shoot (2nd panel) and root vascular tissues (3rd panel). Inlet in upper panel shows HP-treated wild-type SAM with ectopic CLV3::GUS expressing stem cell pool. Bars: (A) 500 µm; (B and D) 200 µm; (E, upper and 2nd panel) 500 µm. (E, 3rd panel) 200 µm.

**Figure 5.** Hyperphyllin (HP) is not a cytokinin analog. A, ARR5::GUS activity in Arabidopsis mock-treated wild type (WT+DMSO), wild type treated with 30 µM HP (WT+HP) and mock-treated *amp1-1* (*amp1*DMSO) at 10 DAG grown in liquid medium. B, Shoot phenotypes of mock-treated (WT+DMSO), 30 µM HP-treated (WT+HP) and 25 µM *trans*-zeatin-treated (Wt+zeatin) wild type plants at 10 DAG grown in liquid medium. C, Effects of HP and *trans*-zeatin on leaf number in wild-type seedlings grown for 10 days in liquid medium containing the indicated concentrations of the compounds. Values are means ± SE (n ≥ 30). Bars: 1 mm.
**Figure 6.** Hyperphyllin (HP)-treated Arabidopsis wild-type and amp1 plants have significantly overlapping transcriptional responses. A, Venn diagram showing the number of overlapping and non-overlapping genes that exhibited altered expression in amp1-13 (blue) and in wild type in response to HP treatment (green) and percentage of total misregulated genes from the two samples falling in the overlapping group. B, Diagram displaying the fraction of hormone-specific marker genes misregulated in HP-treated wild type and in amp1-13. ACC: ethylene (1-amino-cyclopropane-1-carboxylic acid); CK: cytokinin (trans-zeatin); BL: brassinosteroid (brassinolide); IAA: auxin (indole-3-acetic acid); ABA: abscisic acid; MJ: jasmonic acid (methyl jasmonate).

**Figure 7.** Hyperphyllin responses are substantially alleviated in the amp1 mutant background. A, Quantification of leaf number in the indicated genotypes at 10 DAG grown in liquid medium containing either only the solvent DMSO or 30 μM HP. Values represent means ± SE (n ≥ 15) Statistical significance between treated and untreated samples of the same genotype were calculated with the Student’s two-tailed t-test; the resulting P values are shown. B, Scanning electron micrographs of SAM areas of amp1-1 seedlings grown for 10 days in liquid medium containing either only the solvent DMSO or 30 μM HP. C, Quantification of SAM area from scanning electron micrographs of mock-treated (DMSO) 30 μM HP-treated amp1-1 seedlings (HP) Values are means ± SE (n ≥ 5). D, Comparison of GUS activities of indicated reporter lines in mock-treated (amp1-1+DMSO) and 30 μM HP-treated (amp1-1+HP) amp1-1 seedlings at 10 DAG grown in liquid medium. Right WUS::GUS panel shows plants with multiple OCs including the frequency of appearance (n ≥ 20). E, Venn diagram showing the number of overlapping and non-overlapping genes that were HP-regulated in wild type (blue), constitutively misregulated in amp1-13 (green) and HP-regulated in
amp1-13 (red) based on the transcriptomic analysis using Arabidopsis Gene 1.1 ST
Array Strips. Bars: (B) 50 µm.

**Figure 8.** Hyperphyllin (HP) elevates PHAVOLUTA protein levels in wild type. A, Immunoblotting of protein extracts of 10-day-old 35S::PHV-MYC and 35S::PHV-YFP seedlings in the indicated genetic backgrounds grown in liquid medium containing only the solvent DMSO (-), 30 µM HP (HP) or 30 µM A1 (A1). Upper panel autoradiogram: PHV-MYC detection using an anti-MYC antibody. Lower panel autoradiogram: PHV-YFP detection using an anti-GFP antibody. Coomassie blue staining of the membrane is shown as a loading control (CBS). Normalized relative signal intensities are indicated. B, qPCR analysis of PHV expression in 10-d-old seedlings of the indicated lines. The SE was calculated from three biological replicates. UBC was used as an internal control.
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B

WT  amp1  amp1
35S::GCP II:MYC

C

amp1  35S::GCP II-MYC  amp1

kDa  1  2  3
130  
100  
70   
55   
40   
anti-MYC
loading

D

WT+DMSO  WT+PBDA  pt +DMSO
**Figure 1.** Analysis of the degree of functional conservation between Arabidopsis AMP1 and human GCPII. A, List of amino acid (aa) residues defining the indicated functional features in HsGCPII (Mesters et al., 2006) and their corresponding aa residues in AMP1 based on a protein sequence alignment shown in Supplemental Fig. S1. Divergent aa residues are indicated in green. B, Shoot phenotypes of Arabidopsis wild type, 35S::GCPII-MYC amp1-1 line 2 and amp1-1 at 14 days after germination (DAG). C, Immunoblotting of protein extracts of 2-week-old seedlings of the indicated 35S::GCPII-MYC amp1-1 lines using α-MYC antibody for GCPII-MYC detection. Upper panel: autoradiogram, lower panel: Ponceau S staining as a loading control. D, Shoot phenotypes of Arabidopsis seedlings of the indicated genotypes grown for 10 days in liquid medium containing either only the solvent DMSO or 50 µM PBDA, WT, wild type; pt, the amp1 allele primordia timing. Bars: (B) 5 mm; (D) 1 mm.
**Figure 2.** Hyperphyllin (HP) treatment phenocopies *amp1*-associated shoot defects. A, E and I, Comparison of Arabidopsis wild-type (A and E) and *amp1-1* (I) seedlings grown for 10 days in liquid medium containing either only the solvent DMSO (A and I) or 30 μM hyperphyllin (HP, E). B, F and J, Comparison of CYCB1;1::GUS activity in Arabidopsis wild-type (B and F) and *amp1-1* (J) seedlings grown for 10 days in liquid medium containing either only the solvent DMSO (B and J) or 30 μM HP (F). C, G and K, Median longitudinal SAM sections of Arabidopsis wild-type (C and G) and *amp1-1* (K) seedlings grown for 10 days in liquid medium containing either only the solvent DMSO (C and K) or 30 μM HP (G). Scanning electron micrographs of SAM areas of Arabidopsis wild-type (D and H) and *amp1-1* (L) seedlings grown for 10 days in liquid medium containing either only the solvent DMSO (D and L) or 30 μM HP (H). M, Quantification of leaf number in mock-treated wild type (WT+DMSO), wild type treated with 30 μM HP (WT+HP) and mock-treated *amp1-1* (*amp1-1+DMSO*) at 10 DAG grown in liquid medium. Values are means ± SE (n ≥ 30). N, Quantification of SAM size from median longitudinal sections of mock-treated wild type (WT+DMSO), wild type treated with 30 μM HP (WT+HP) and mock-treated *amp1-1* (*amp1-1+DMSO*) at 10 DAG grown in liquid medium. Values are means ± SE (n ≥ 3). Bars: (A, E and I) 2 mm; (B, F and J) 1 mm; (C, D, G, H, K and L) 50 μm.
Figure 3. Structure-activity relationship of hyperphyllin (HP) and its analogues. A, Chemical structures of HP and its active (A1, A2, A3) and inactive (I1, I2, I3) analogues. B, Effects of HP and analogues A1 and A2 on leaf number in Arabidopsis wild-type seedlings grown for 10 days in liquid medium containing the indicated concentrations of the compounds. Values are means ± SE (n ≥ 10). C, Correlation of structure-based hierarchical clustering of HP and its analogues with their respective effects on the number of leaves formed at a concentration of 50 µM. Values are means ± SE (n ≥ 30). Hierarchical clustering was generated by the ChemMine clustering tool (see materials and methods).
**Figure 4.** Shoot apical meristem (SAM) marker activities in Arabidopsis hyperphyllin (HP)-treated wild-type plants are highly reminiscent to those of *amp1*. Comparison of SAM marker activities in Arabidopsis mock-treated wild type (WT+DMSO), wild type treated with 30 µM HP (WT+HP) and mock-treated *amp1-1* (*amp1*DMSO) at 10 DAG grown in liquid medium. A, pKLU::GUS activity. B, WUS::GUS activity. C, Percentage of plants showing ectopic WUS expression foci in the SAM (n ≥ 100). D, 30 µM HP-treated wild-type and mock-treated *amp1-1* seedling with ectopic WUS expression foci in the SAM. E, CLV3::GUS activity in the SAM (upper panel), in the shoot (2nd panel) and root vascular tissues (3rd panel). Inlet in upper panel shows HP-treated wild-type SAM with ectopic CLV3::GUS expressing stem cell pool. Bars: (A) 500 µm; (B and D) 200 µm; (E, upper and 2nd panel) 500 µm. (E, 3rd panel) 200 µm.
A) WT+DMSO, WT+HP, amp1+DMSO

B) WT+DMSO, WT+HP, WT+zeatin

C) Leaf number vs. concentration (µM)

conc: 0 10 20 30 40 50 60 70 80

Leaf number: 3 5 7 9 11

HP, zeatin
Figure 5. Hyperphyllin (HP) is not a cytokinin analogue. A, ARR5::GUS activity in Arabidopsis mock-treated wild type (WT+DMSO), wild type treated with 30 µM HP (WT+HP) and mock-treated amp1-1 (amp1+DMSO) at 10 DAG grown in liquid medium. B, Shoot phenotypes of mock-treated (WT+DMSO), 30 µM HP-treated (WT+HP) and 25 µM trans-zeatin-treated (Wt+zeatin) wild type plants at 10 DAG grown in liquid medium. C, Effects of HP and trans-zeatin on leaf number in wild-type seedlings grown for 10 days in liquid medium containing the indicated concentrations of the compounds. Values are means ± SE (n ≥ 30). Bars: 1 mm.
A. Venn diagram showing misregulated genes in response to HP-treatment (918 genes) and in amp1-13 (608 genes) with 345 genes overlapping.

B. Bar graph showing the percentage of overlapping genes for different treatments: ACC, CK, BL, IAA, ABA, and MJ, comparing WT+HP and amp1-13.
Figure 6. Hyperphyllin (HP)-treated Arabidopsis wild-type and amp1 plants have significantly overlapping transcriptional responses. A, Venn diagram showing the number of overlapping and non-overlapping genes that exhibited altered expression in amp1-13 (blue) and in wild type in response to HP treatment (green) and percentage of total misregulated genes from the two samples falling in the overlapping group. B, Diagram displaying the fraction of hormone-specific marker genes misregulated in HP-treated wild type and in amp1-13. ACC: ethylene (1-amino-cyclopropane-1-carboxylic acid); CK: cytokinin (trans-zeatin); BL: brassinosteroid (brassinolide); IAA: auxin (indole-3-acetic acid); ABA: abscisic acid; MJ: jasmonic acid (methyl jasmonate).
Figure 7. Hyperphyllin responses are substantially alleviated in the amp1 mutant background. A, Quantification of leaf number in the indicated genotypes at 10 DAG grown in liquid medium containing either only the solvent DMSO or 30 μM HP. Values represent means ± SE (n ≥ 15) Statistical significance between treated and untreated samples of the same genotype were calculated with the Student’s two-tailed t-test; the resulting P values are shown. B, Scanning electron micrographs of SAM areas of amp1-1 seedlings grown for 10 days in liquid medium containing either only the solvent DMSO or 30 μM HP. C, Quantification of SAM area from scanning electron micrographs of mock-treated (DMSO) 30 μM HP-treated amp1-1 seedlings (HP) Values are means ± SE (n ≥ 5). D, Comparison of GUS activities of indicated reporter lines in mock-treated (amp1-1+DMSO) and 30 μM HP-treated (amp1-1+HP) amp1-1 seedlings at 10 DAG grown in liquid medium. Right WUS::GUS panel shows plants with multiple OCs including the frequency of appearance (n ≥ 20). E, Venn diagram showing the number of overlapping and non-overlapping genes that were HP-regulated in wild type (blue), constitutively misregulated in amp1-13 (green) and HP-regulated in amp1-13 (red) based on the transcriptomic analysis using Arabidopsis Gene 1.1 ST Array Strips. Bars: (B) 50 μm.
### Table A

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### Graph B

Relative PHV mRNA levels

- DMSO
- HP

35S::PHV-MYC
35S::PHV-YFP
Figure 8. Hyperphyllin (HP) elevates PHAVOLUTA protein levels in wild type. A, Immunoblotting of protein extracts of 10-day-old 35S::PHV-MYC and 35S::PHV-YFP seedlings in the indicated genetic backgrounds grown in liquid medium containing only the solvent DMSO (-), 30 μM HP (HP) or 30 μM A1 (A1). Upper panel autoradiogram: PHV-MYC detection using an anti-MYC antibody. Lower panel autoradiogram: PHV-YFP detection using an anti-GFP antibody. Coomassie blue staining of the membrane is shown as a loading control (CBS). Normalized relative signal intensities are indicated. B, qPCR analysis of PHV expression in 10-d-old seedlings of the indicated lines. The SE was calculated from three biological replicates. UBC was used as an internal control.


Nogué F, Grandjean O, Craig S, Dennis ES, Chaudhury AM (2000a) Higher levels of cell proliferation rate and cyclin CycD3 expression in the Arabidopsis amp1 mutant. Plant Growth Regulation 32: 275-283


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