ORIGINAL ARTICLE

Establishment of an Agrobacterium mediated transformation protocol for the detection of cytokinin in the heterophyllous plant *Hygrophila difformis* (Acanthaceae)

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Abstract

Key message **This is the first report of a highly efficient *Agrobacterium tumefaciens*-mediated transformation protocol for Acanthaceae and its utilization in revealing important roles of cytokinin in regulating heterophylly in *Hygrophila difformis*.**

Abstract Plants show amazing morphological differences in leaf form in response to changes in the surrounding environment, which is a phenomenon called heterophylly. Previous studies have shown that the aquatic plant *Hygrophila difformis* (Acanthaceae) is an ideal model for heterophylly study. However, low efficiency and poor reproducibility of genetic transformation restricted *H. difformis* as a model plant. In this study, we reported successful induction of callus, shoots and the establishment of an efficient stable transformation protocol as mediated by *Agrobacterium tumefaciens* LBA4404. We found that the highest callus induction efficiency was achieved with 1 mg/L 1-Naphthaleneacetic acid (NAA) and 2 mg/L 6-benzyladenine (6-BA), that efficient shoot induction required 0.1 mg/L NAA and 0.1 mg/L 6-BA and that high transformation efficiency required 100 μM acetosyringone. Due to the importance of phytohormones in the regulation of heterophylly and the inadequate knowledge about the function of cytokinin (CK) in this process, we analyzed the function of CK in the regulation of heterophylly by exogenous CK application and endogenous CK detection. By using our newly developed transformation system to detect CK signals, contents and distribution in *H. difformis*, we revealed an important role of CK in environmental mediated heterophylly.

Keywords *Agrobacterium tumefaciens* · Cytokinin · Heterophylly · *Hygrophila difformis* · Transformation · Leaf form

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Introduction

Plants have rich morphological diversity among different species. The leaves of some plant species are able to change with their growing environment and this morphological variation is called heterophylly (Zotz et al. 2011). Most known

examples of heterophylly are found in aquatic and amphibious plants, in which submerged leaves are always dissected, thin, narrow and lack stomata, whereas terrestrial leaves are simple, thicker, expanded and have abundant stomata (Wells and Pigliucci 2000; Wanke 2011; Li et al. 2017, 2019a). Heterophylly is an extreme example of the leaf shape changes that occur in all plants in order to optimize physiological function to the environment, so heterophylly provides an ideal process to understand the mechanism by which plants acclimate their growth to withstand environmental changes (Kuwabara et al. 2003; Wanke 2011; Kim et al. 2018).

Heterophylly is observed in a large number of aquatic plants, including those in Brassicales, Euphorbiales, Marsileales, Myrtiflorae, Nymphaeales, Ranales and Tubiflorae (Cook 1969; Deschamp and Cooke 1984; Lin and Yang 1999; Kuwabara et al. 2001; Titus et al. 2001; Nakayama et al. 2014; Li et al. 2017). Many environmental factors, like humidity, temperature, CO_2 and light are involved in regulating heterophylly. For example, high humidity can significantly change the leaf shape of *Hygrophila difformis* from serrated to highly dissected forms (Li et al. 2017). Low temperature or high CO_2 can induce deep-lobed aquatic leaves of *Ranunculus flabellaris* in terrestrial conditions (Johnson 1967; Bristow 1969). High concentrations of CO_2 can also induce the aquatic phenotypes of *Marsilea vestita* (Bristow and Looi 1968) while lower free CO_2 stimulates the development of floating leaves of *Nuphar variegate* (Titus et al. 2001). Light photoperiod is also a factor that can regulate leaf forms, as the divided leaf shape of *Ranunculus aquatilis* can be induced by short photoperiods (Cook 1969). Blue light induced the terrestrial phenotype of *Marsilea quadrifolia* under submerged conditions (Lin and Yang 1999) and high light density can induce the submerged leaf form of *Rorippa aquatica* under terrestrial conditions (Nakayama et al. 2014). In addition, a recent study has showed that cold or hypoxic conditions can induce the aquatic leaf shape formation in *Ranunculus trichophyllus* (Kim et al. 2018).

Phytohormones are also known to have a role in heterophylly, including abscisic acid (ABA), ethylene, gibberellin (GA) and others (Lin and Yang 1999; Kuwabara et al. 2003; Jackson 2007; Wanke 2011; Nakayama et al. 2017). In some studies, ethylene and GA seems to be antagonists of ABA and have antagonistic effects on the transition to the terrestrial form. In *Callitriche heterophylla*, exogenous GA induced slender aquatic leaves, while ABA induced submerged shoots to produce expanded, land-form leaves (Deschamp and Cooke 1984). When exogenous ethylene was applied to terrestrial shoots of *Ludwigia arcuate*, narrow aquatic leaves formed, while treatment with ABA induced rounded terrestrial leaves under submerged conditions (Kuwabara et al. 2003). ABA/ethylene signaling are also key factors for the regulation of heterophylly in *R. trichophyllus*, as compared to terrestrial leaves, aquatic leaves have higher

levels of ethylene but lower levels of ABA (Kim et al. 2018). Transcriptome analyses of submerged and floating leaves suggested that auxin plays an important role in regulating heterophylly in *Potamogeton octandrus* (He et al. 2018; Li et al. 2019b).

In addition, cytokinin (CK) plays key roles in a wide variety of processes of plant growth and development, such as cell division, differentiation, leaf senescence, nutrient mobilization, seed germination and pathogen responses (Su et al. 2011; Efroni et al. 2013; Shanks et al. 2018; Takatsuka and Umeda 2019). Studies have shown that CK levels change with environmental conditions in *R. aquatica* and that CK is involved in prolonged morphogenetic activity of leaf margin (Shani et al. 2010; Nakayama et al. 2014).Despite these hints that CK has a role in the formation of dissected leaves, the question that CK influences heterophylly has yet to be addressed experimentally.

Even though the phenomenon of heterophylly is widely seen in aquatic plants, the molecular mechanism behind it is poorly understood due to the lack of suitable models (Nakayama et al. 2014, 2017; Kim et al. 2018). *Hygrophila difformis* (Acanthaceae) is an aquatic plant which shows well-defined phenotypic differences between terrestrial and submerged leaves (Fig. 1). Previous studies have shown that this aquatic plant is an ideal model for the study of heterophylly due to its distinct leaf features, sensitivity to environment, convenience in culture and propagation (Li et al. 2017), as well as advantages for the study of photosynthetic acclimation of heterophyllous plants (Horiguchi et al. 2019). However, the use of *H. difformis* as a model plant was restricted by the difficulties with tissue culture, together with the relative low efficiency and poor reproducibility of genetic transformation. Indeed, there were no reports of efficient transformation protocol for the family Acanthaceae, suggesting that the family might be recalcitrant to transformation. In this study, we reported successful and efficient stable transformation of *H. difformis* using the *Agrobacterium tumefaciens* strain LBA4404 by optimizing each step of the process. This is the first report of highly efficient *Agrobacterium tumefaciens*-mediated transformation in Acanthaceae, which will provide important reference to other plants in the family. Using exogenous CK application, endogenous CK detection and this transformation system, we determined that cytokinin has a significant function in the regulation of heterophylly in *H. difformis*.

Materials and methods

Plant materials and growth condition

Hygrophila difformis plants were grown in growth chambers under 16 h daylength with the temperature set at 23 °C, with
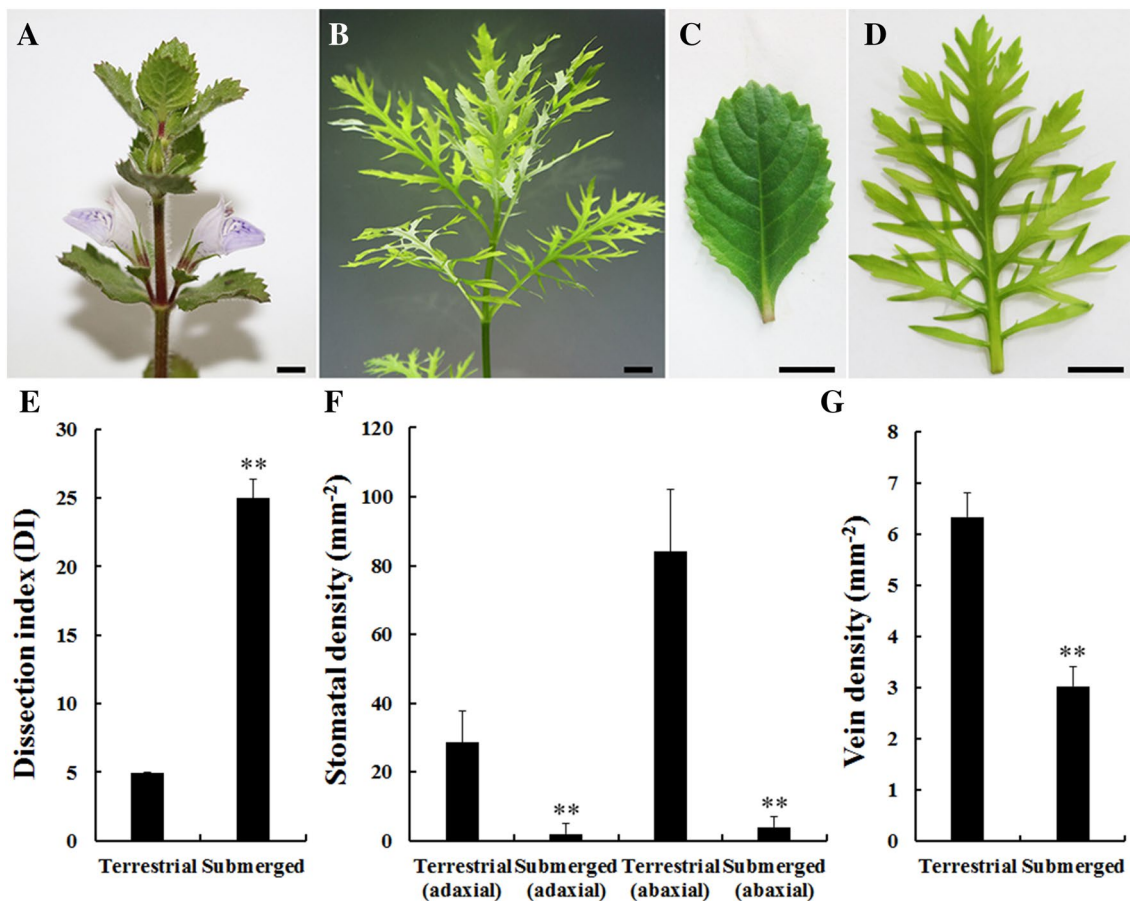
Fig. 1 Phenotype of *H. difformis* under terrestrial and submerged conditions. **a** A plant grown in the terrestrial environment. **b** A plant grown in the submerged environment. **c** Terrestrial leaf. **d** Submerged leaf. **e** Dissection index (DI) of P6 leaves from plants grown under terrestrial or submerged conditions. Data are mean ± SD ($n = 3$). ** indicates significant difference to the terrestrial, $P < 0.01$ (Student's t test). **f** Comparison of stomatal density in leaves grown under ter- restrial or submerged conditions: the number of stoma on abaxial and adaxial sides per unit area. Data are mean ± SD ($n = 10$). ** indicates significant difference to the terrestrial, $P < 0.01$ (Student's t test). **g** Comparison of minor vein density in leaves grown under ter- restrial or submerged conditions: vein length per unit area. Data are mean ± SD ($n = 10$). ** indicates significant difference to the terres- trial, $P < 0.01$ (Student's t test). Bars =1 cm in (**a–d**).

a white light flux density of 60 μmol/m^2/s. For humidity treatment, the humidity was controlled at a 60% or 30% RH (Relative Humidity), 16 h daylength with the temperature set at 26 °C. For high/low temperature condition, plants in the chamber were grown at 20 or 26 °C, with humidity at 60% RH. Most of the plants used for morphological observations and phytohormone application experiments were grown for 1.5–2 months. For phenotypic analysis, the P6 leaf taken from plants grown under constant conditions including the hormonal treatments.

Morphological observation

All light microscopic observations were performed on a Sunny EX20 light microscope. Images were captured with a ToupCam TP605100A digital camera using the MvImage media software (ToupCam) in BMP format at a resolution of 2592 × 1944. All measurements were performed using ImageJ 1.47v (https://rsb.info.nih.gov/ij/). Leaf form com- plexity was estimated by "dissection index (DI)", calculated as (leafperiphery)/$\sqrt{\text{leafarea}}$. For quantification of stoma- tal density, experiments were performed as described pre- viously (Li et al. 2017). For quantification of vein density, experiments were performed as described previously (Sack et al. 2012). Quantifications of stomatal density and vein density were conducted from leaves of at least ten independ- ent plants.

Phytohormones treatment and detection

Plants were treated with a synthetic cytokinin (6-BA) or cytokinin inhibitor (lovastatin). For the hormone and antagonist treatments, a 100-μL drop of each solution was applied to the shoot apex every day for 1.5 months. Plants

were treated with 10 μM 6-BA or 5 μM lovastatin all in 0.1% (*w/v*) ethanol; the control solution comprised 0.1% (*w/v*) ethanol alone. Every treatment had 3 replicates. After 1.5 months, mature leaves were harvested and their morphology analysed. For extraction and quantification of endogenous trans-Zeatin (tZ, bioactive form of cytokinin), shoots (including leaf primordia) were sampled and assessed using ultraperformance liquid chromatography coupled with tandem quadruple mass spectrometry as described previously (Nakayama et al. 2014; Li et al. 2016). Quantitative RT-PCR analysis was conducted as previously (Li et al. 2017) and using primers shown in Table S1. Experiments were conducted in triplicate from three independent plants.

Callus induction and shoot induction

To generate callus from leaf fragments, young leaves of *H. difformis* were sterilized for 15 s in 70% ethanol followed by 4 min in 0.1% HgCl$_2$. Sterilized leaf fragments were placed on Murashige and Skoog (MS) medium containing 8 g/L agar and 2% (*w/v*) sucrose at pH 5.8 and growth at 24 °C. To establish the most effective hormone combination for callus induction, callus formation was compared following 1 month culture of leaf disks on the above medium with phytohormones, 6-benzyladenine (6-BA) and 1-Naphthaleneacetic acid (NAA) in all combinations of five different combinations (0.2, 0.4, 1, 2, 4 mg/L). Similarly, to establish the hormone concentrations most effective for shoot induction, fresh calli were placed on the same medium for about 1 month with all combinations of four different concentrations (0.1, 0.25, 0.5, 1 mg/L) of 6-BA and NAA. Callus induction and shoot induction efficiencies in this study are presented by means ± SDs.

Agrobacterium strain and vector preparation

The TCS element was synthesized and separately inserted into the binary vector pKGWFS7.0 (https://www.transgen.com.cn/) (Fig. S1) followed methods described in previous studies (Müller and Sheen, 2008; Li et al. 2017). *Agrobacterium tumefaciens* strain LBA4404 (https://www.transgen.com.cn/) was used as the host strain for the constructed TCS vector. Transformed Agrobacterium were then cultured on solid YEB medium [0.6% (*m/v*) yeast extract, 0.5% (*m/v*) tryptone, 2 mM MgSO$_4$, 0.5% (*m/v*) glucose and 0.6% (*m/v*) agar at pH 7.0] supplemented with 24.30 μM rifampicin and 246.75 μM spectinomycin and grown at 28 °C for 2 days. The Agrobacterium was then harvested and shaken in liquid YEB medium until an OD600 of 0.5 was reached.

Co-cultivation

For infection, fresh calli were dissected into small fragments (5 mm in diameter) and immersed into Agrobacterium suspension for 30 min. For co-cultivation, petri dishes containing sterilized filter paper (6–8 layers) were pre-soaked in liquid co-cultivation medium. Infected calli were transferred onto the filter paper and incubated for 3 days in the dark at 23 ± 1 °C. Each plate contained 8–10 callus and every experiment had 3 replicates.

To investigate the effect of acetosyringone (AS) on transformation efficiency, the infected calli were transferred to co-cultivation media at pH 5.2 (MS medium with 4.58 mM 4-Morpholine Ethane Sulfonic Acid (MES), 1 mg/L NAA and 2 mg/L 6-BA) with different concentrations of AS (0, 50, 100 and 200 μM). To evaluate the effect of pH, co-cultivation medium (MS medium with 4.58 mM MES, 1 mg/L NAA and 2 mg/L 6-BA and 100 μM AS) at different pH levels (4.8, 5.2 and 5.6) were prepared.

Regeneration and selection

Co-cultivated calli were transferred to regeneration medium [MS medium with 1 mg/L NAA and 2 mg/L 6-BA, 1% (*m/v*) sucrose, 0.6% (*m/v*) agar and 0.63 mM cefotaxime, pH 5.8] for 4 days. Calli were then transferred into selection medium [MS medium with 1 mg/L NAA and 2 mg/L 6-BA, 1% (*m/v*) sucrose, 0.6% (*m/v*) agar and 0.63 mM cefotaxime, 144.36 μM geneticin (G418) at pH 5.8]. This selection process lasted for at least 1.5 month. Surviving calli were transferred to fresh medium weekly. Both regeneration and selection cultivation were conducted under 25 ± 1 °C, with a light density of 85 μmol/m^2/s and a 16 h light photoperiod.

Shoot induction and proliferation

Green callus was transferred from selection medium to shoot induction medium [MS medium with 0.1 mg/L NAA and 0.1 mg/L 6-BA, 8 g/L Agarose, and 2% (*w/v*) sucrose] and incubated at 25 ± 1 °C, with a light density of 85 μmol/m^2/s and a 16 h light photoperiod until multiple shoots start to appear. Plantlets were then moved to tissue culture jars (200 mL) [MS medium with 8 g/L Agarose and 2% (*w/v*) sucrose] and propagated vegetatively.

PCR and GUS identification

For PCR analysis, total genomic DNA was extracted from growing plants using a Cetyltrimethyl Ammonium Bromide (CTAB) protocol (Clarke 2009). The extracted DNA was used as templates for PCR analysis to test for presence of the TCS element using specified primers (Table S1) and PCR amplification was performed as follows: initial denaturation

at 94 °C for 5 min, then 35 cycles of denaturation at 94 °C for 30 s, annealing at 57 °C for 30 s, extension at 72 °C for 1 min and a final extension at 72 °C for 5 min. To detect GUS reporter activity, tissues were vacuum infiltrated in staining buffer [0.1 M potassium phosphate buffer, 5 mM ethylenediaminetetraacetic acid (EDTA), 0.5 mM potassium ferric cyanide and ferrous cyanides, and 0.5 mg/mL 5-bromo-4-chloro-3-indolyl glucuronide cyclohexylammonium salt (X-Gluc)] for 30 min, stained overnight at 37 °C and cleared through an incremental ethanol series (Li et al. 2017).

Results

The optimization of phytohormones for callus induction

The balance between two phytohormones, auxin and cytokinin, determines the state of differentiation, and exogenous application of auxin and cytokinin has been shown to induce callus in various plant species (Skoog and Miller 1957; Su et al. 2011; Ikeuchi et al. 2013). To explore the suitable proportions of phytohormones for callus induction, we altered the concentrations of both auxin (NAA) and cytokinin (6-BA) in the callus induction medium. Our results revealed that low concentration (0.2 mg/L) of 6-BA and NAA failed to induce the formation of callus, while increasing the concentration of 6-BA and NAA promoted callus induction efficiency (Fig. 2a). The highest efficiency of callus induction was achieved with 1 mg/L NAA and 2 mg/L 6-BA, with 83.3% of explants forming callus.

The process of callus formation in *H. difformis* followed a characteristic series of morphological changes. From the young, fresh leaf disks that were used as explants (Fig. 2b) the first observable change was the formation of sporadic bulges around the edges of the leaf disk (Fig. 2c). After a few weeks, callus surrounded the edges of the leaf disk and then gradually covered the whole explant (Fig. 2d, e). When callus grew larger, it was separated into small pieces, which, after being moved to fresh medium, formed green, loose callus (Fig. 2f).

The optimization of phytohormones for shoot induction.

For shoot induction, we use different combinations of NAA and 6-BA in the medium. Our results revealed that low concentration of 6-BA (0.1 mg/L) successfully induced the formation of shoots, while increasing the concentration of 6-BA from 0.1 to 1 mg/L reduced the shoot induction efficiency (Fig. 3a). The highest efficiency of shoot induction was achieved with 0.1 mg/L 6-BA and have an efficiency above 90% of shoots forming. We also found that changing the concentration of NAA from 0.1 to 1 mg/L in the induction medium only had little effects on shoot induction (Fig. 3a).

The process of shoot induction in *H. difformis* occurs through defined changes in the loose green callus that were used as explants (Fig. 3b). First, bud points formed on the surface of callus, together with the color changing to dark green (Fig. 3c). After few weeks, buds developed into small shoots and a few plants formed (Fig. 3d). After about 3 weeks of cultivation, all callus disappeared and was replaced by the proliferation of many strong plants that could be transplanted (Fig. 3e).

The optimization of AS and pH for transformation efficiency of *H. difformis*.

AS has been previously shown to facilitate in the transformation of a large number of plant species, including both monocotyledons and dicotyledons (Singh and Prasad 2016). The pH of co-cultivation medium also affected gene transformation efficiency in a few species (Chhabra et al. 2011; Yang et al. 2018). To development an efficient transformation system of *H. difformis*, we first assessed the effect of AS (Fig. 4a). We found that AS significantly improved transformation efficiency, with the highest transformation efficiency $(15.6 \pm 1\%)$ with 100 µM AS, significantly higher than the control $(1.8 \pm 0.5\%)$.

We next determined the effect of pH of the co-cultivation media on the transformation efficiency (Fig. 4b). However, no significant difference was found following these treatments, suggested that, in the range tested, pH does not influence the transformation of *H. difformis*.

To test for the presence of the TCS transgene in the putatively transformed plants, we then performed PCR amplification on DNA isolated from three randomly selected transgenic lines (T1, T2 and T3). All showed an amplification product (Fig. 4c). GUS staining showed that GUS was expressed in callus, induced shoots and whole plants (Fig. 4d–g). These results indicate the success of our transformation and regeneration protocol, summarized in Fig. 5.

The detection of endogenous CK in heterophylly of *H. difformis*

Plant hormones are known to be involved in many heterophyllous plants, however, little is known about the function of CK in the regulation of heterophylly (Kuwabara et al. 2003; Kim et al. 2018). Previous studies have demonstrated that changes in the endogenous CK levels in shoots affect leaf complexity in both terrestrial and aquatic species with compound leaves (Shani et al. 2010; Nakayama et al. 2014). We therefore proposed that CK level might be also involved in the leaf form response

Fig. 2 The effect of different concentration of 6-BA and NAA on callus induction of *H. difformis*. **a** The efficiency of callus induction in media with different combination of 6-BA and NAA. Note that the highest efficiency of callus induction was achieved with 1 mg/L NAA and 2 mg/L 6-BA. Data are mean ± SD (*n* = 3). **b–f** The steps of callus formation in *H. difformis*. The black arrowhead indicate where new callus occurred on the leaf margin. Bars = 1 mm in (**b–f**)
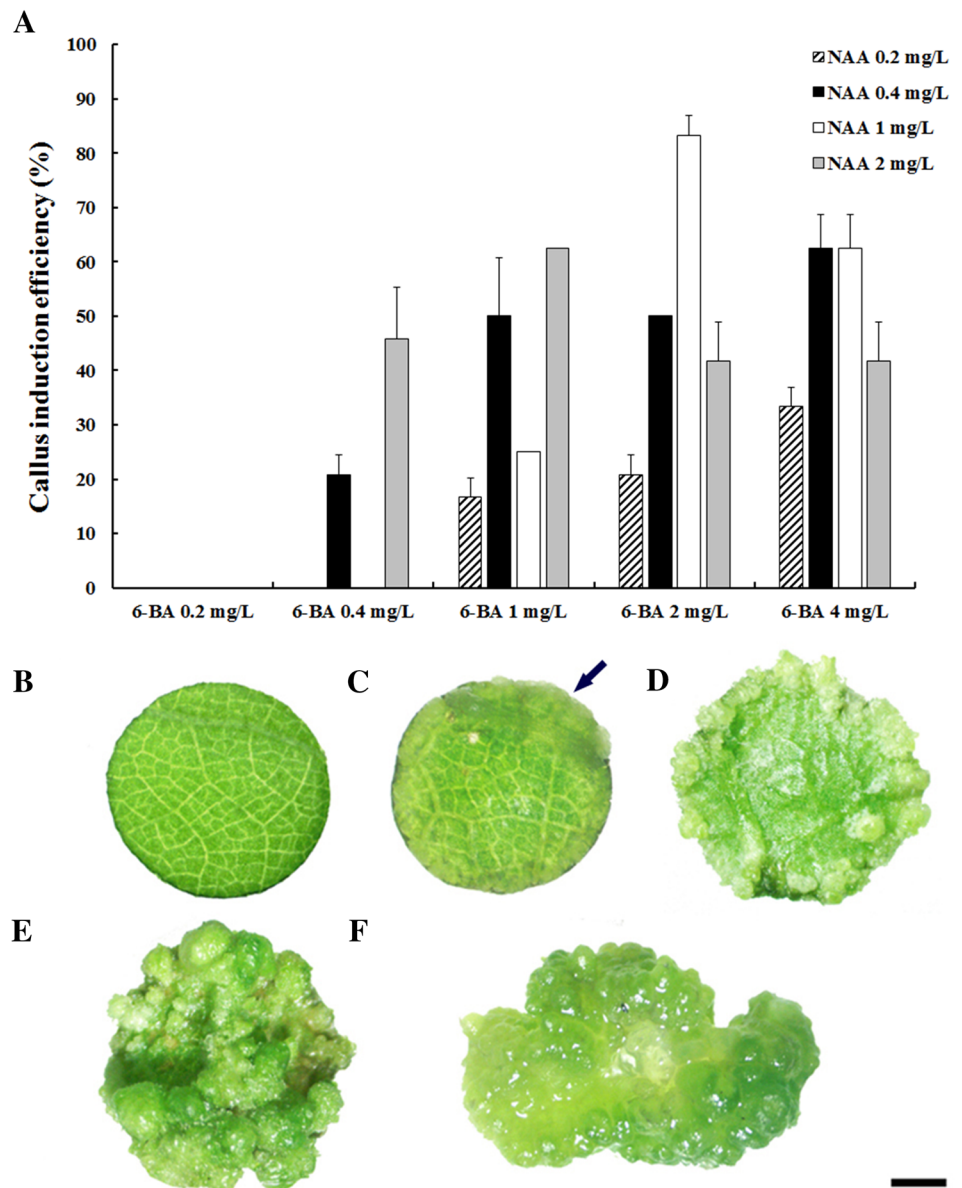
to environmental changes observed in *H. difformis*. To explore whether the phenotype of *H. difformis* changed with environmental factors, we first analyzed the effect of temperature on leaf form. We found that plants grown in 26 °C, 60% RH conditions have deeply dissected leaves and high DI (Fig. 6a, d), a form which usually occurred in the submerged conditions (Fig. 1). However, plants grown in 20 °C, 60% RH conditions (Fig. 6b) have simple leaves with serrated margins and low DI (Fig. 6d), suggesting an important role of temperature in the regulation of leaf form. We then analyzed the effect of humidity on the heterophylly of leaf form and found that plants grown in 26 °C, 60% RH conditions have deeply dissected leaves and high DI (Fig. 6a, d), while plants grown in 26 °C, 30% RH conditions (Fig. 6c) have simple leaves with serrated

margins and low DI (Fig. 6d), suggesting an important role of humidity in the regulation of leaf form.

We then measured the endogenous CK in leaf primordia forming under different conditions. We found that the bioactive form of CK, in shoots was significantly lower at 26 °C, 60% RH condition than at 20 °C, 60% RH conditions or 26 °C, 30% RH conditions (Fig. 6e), suggesting that the endogenous content of CK might be a restrictive factor of complex leaf form. To investigate the expression level of the gene involved in CK degradation, we analysed the expression level of the ortholog of *CYTOKININ DEHYDRO-GENASE 3* (*CKX3*), which encodes the enzyme involved in the degradation of CK. The gene was cloned and identified in *H. difformis*, named as *HdCKX3* and submitted to NCBI (MN585732). The results of qRT-PCR showed that

Fig. 3 The effect of different concentration of 6-BA and NAA on shoot induction of *H. difformis*. **a** The efficiency of shoot induction in media with different combinations of 6-BA and NAA. Note that the highest efficiency of shoot induction was achieved with 0.1 mg/L NAA and 0.1 mg/L 6-BA. Data are mean $\pm$ SD ($n = 3$). **b–e** The steps of shoot formation from callus in *H. difformis*. The black arrowhead in **c** indicates a bud point formed on the surface of callus and the arrowhead in **d** indicates a small shoot on the surface of callus. Bars = 1 mm in (**b–e**)
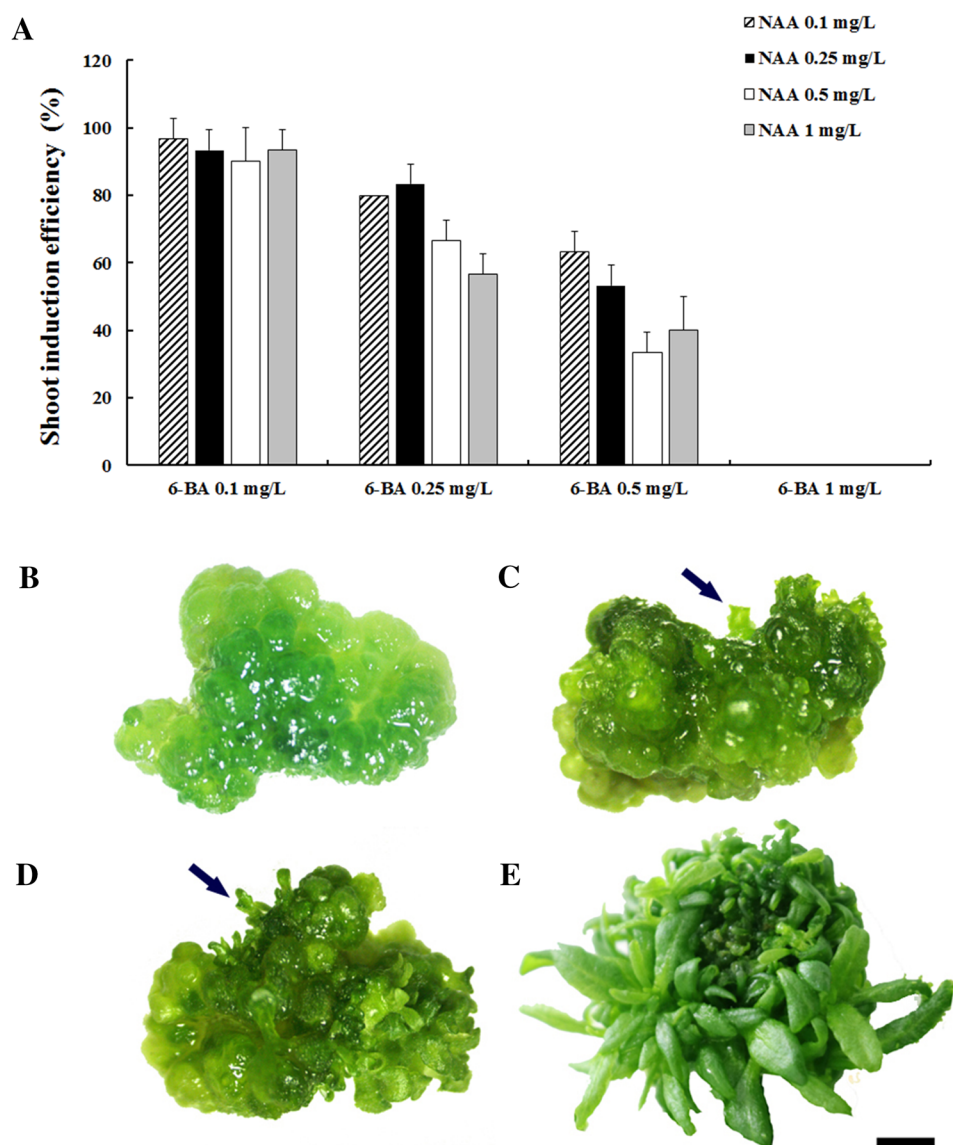
the expression level of *HdCKX3* was significantly changed with temperature and humidity, showing a similar trend to DI and having negative correlation with endogenous CK level (Fig. 6f).

Exogenous CK influences heterophylly in *H. difformis*

To see whether CK is a restrictive factor of compound leaf form, we applied 6-BA and the CK biosynthesis inhibitor lovastatin to the leaf primordia. We found that CK treatment resulted in obviously simplified leaf forms compared with the control plants grown under 26 °C, 60% RH conditions (Fig. 7a, c). However, we did not detect obvious changes in the leaf morphology upon lovastatin treatment (Fig. S2). Plants treated with CK have simple leaves with shallow, serrated margins and low DI (Fig. 7a–d, i), while control

plants have deeply dissected leaves, significantly increased leaf complexity and high DI (Fig. 7a–d, i). As cytokinin has an important function for cell expansion (Holst et al. 2011), we also analyzed the epidermal cell shape of adaxial leaf surface and found that control plants have lobed epidermal cells while plants treated with CK have unlobed and more rectangular cells (Fig. 7c–f).

Considering the important role of CK in vein development (Aloni et al. 2006), we tested the vein density of leaves under CK treatment and found that CK treatment significantly increased minor vein density (Fig. 7g, h, k). Our results also showed that CK has the ability to increase stomatal density of leaves in *H. difformis* (Fig. 7j), which suggested that CK has conserved function of increasing stomatal density in both tomato and *H. difformis* (Farber et al. 2016).
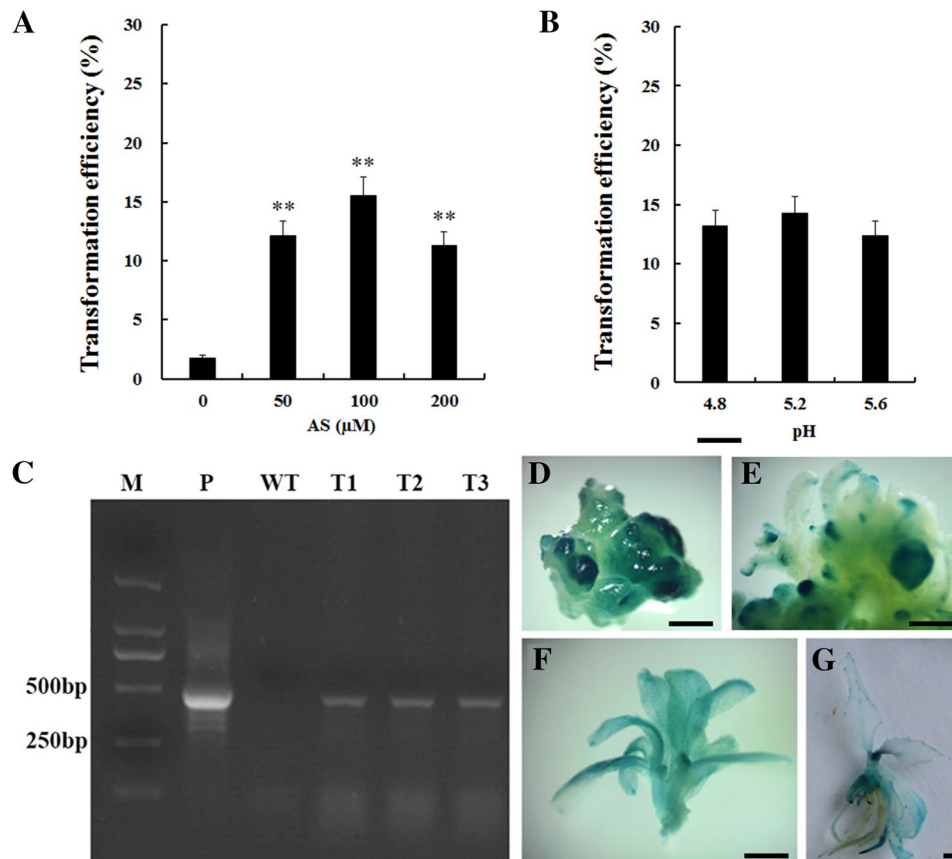
Fig. 4 The effect of AS and pH on transformation of *H. difformis*. **a** The effect of different concentrations of AS on transformation efficiency of *H. difformis*. Data are mean ± SD (*n* = 3). ** indicates significant difference to the control, *P* < 0.01 (Student's *t* test). **b** The effect of different pH on transformation efficiency of *H. difformis*. Data are mean ± SD (*n* = 3). **c** Confirmation of *H. difformis* transformation by PCR analysis. Agarose gel following electrophoresis of (M) 2000 bp DNA ladder marker, with 250 bp and 500 bp indicated;

(P) PCR amplification product using plasmid containing TCS element as template; (WT) PCR amplification product using DNA exracted from wild plants as template. (T1–3) PCR amplification product using DNA exracted from plants transformed with *TCS::GUS* element as template. The expected size of the TCS amplion is 404 bp. **d–g** GUS staining of callus and plants transformed with *TCS::GUS* report construct. **d** Callus; **e** shoot induction; **f** adventitious shoots; **g** mature plants. Bars = 1 mm.
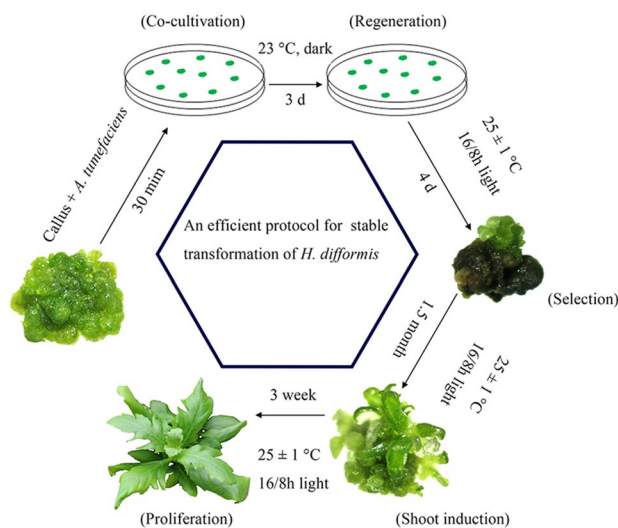

Fig. 5 The process of genetic transformation for *H. difformis* mediated by *A. tumefaciens*

The distribution of CK in leaf development

Although our results suggest an important function of CK in regulating leaf form in *H. difformis*, the question of how CK is involved in the process of leaf development remains. To further analyze the function of CK in the development of *H. difformis*, we employed our optimized transformation protocol to introduce the *TCS::GUS* reporter construct, allowing the dynamic distribution of CK to be detected during leaf development (Müller and Sheen 2008). The *TCS::GUS* line showed dispersed GUS distribution at the initial stage of leaf development (Fig. 8a, e). Magnified views showed that the blue color occurred in most of trichomes on the leaf surface (Fig. 8i). As development progressed, GUS activity gradually became restricted to the veins and hydathodes around leaf margins, with weaker GUS expression distributed through the whole leaf (Fig. 8b, f). Magnified views showed that the blue color occurred in the vascular bundles
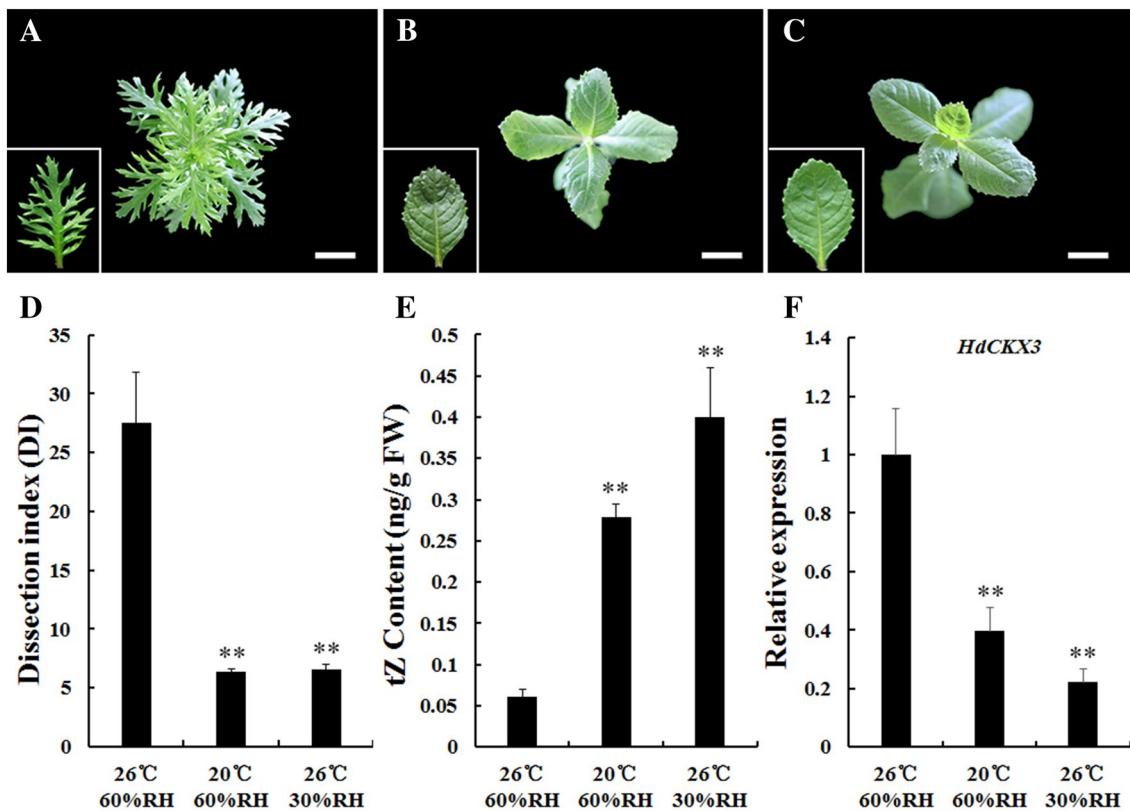
Fig. 6 Endogenous CK and leaf shape are significantly changed with humidity and temperature. **a** Top view of plants grown in 26 °C, 60% RH conditions have deeply dissected leaves. Note that plants grown in this condition was set as control. **b** Top view of plants grown in 20 °C, 60% RH conditions have simple, serrated leaves. **c** Top view of plants grown in 26 °C, 30% RH conditions have simple, serrated leaves. **d** Dissection index (DI) of P6 leaves from plants of different conditions. Data are mean ± SD ($n = 3$). ** indicates significant dif-ference to the control, $P < 0.01$ (Student's t test). **e** The content of *trans*-Zeatin of shoots from plants of different conditions. Data are mean ± SD ($n = 3$). ** indicates significant difference to the control, $P < 0.01$ (Student's t test). (F) The relative expression of *HdCKX3* from shoots from plants of different conditions. Data are mean ± SD ($n = 3$).. ** indicates significant difference to the control, $P < 0.01$ (Student's t test). Bars = 1 cm in (**a**–**c**)

and trichomes on the leaf surface (Fig. 8j). With further leaf development, the levels of GUS activity sharply decreased and become more restricted to trichomes and hydathodes around leaf margins (Fig. 8c, g, k). As leaves became mature, the GUS activity became increasing in the minor veins, hydathodes around the leaf margin and dispersed in the whole leaf (Fig. 8d, h). Magnified views showed that the dispersed blue color mostly occurred in the guard cells and trichomes on the leaf surface (Fig. 8l).

Discussion

An efficient genetic transformation system is a prerequisite for studying gene functions and molecular mechanisms. Many factors are involved in the genetic transformation efficiency of plants, of which one crucial factor is the combination of different phytohormones for callus and shoot induction (Trieu et al. 2000). In numerous Acanthaceae species different concentrations of NAA and 6-BA induce shoot regeneration, with 0.2 mg/L NAA and 2 mg/L 6-BA effective in *Asteracantha longifolia* (Panigrahi et al. 2007), 0.5 µM NAA and 1 µM 6-BA inducing shoots in *Hygrophila spinosa* (Varshney et al. 2009), and 0.2 mg/L NAA and 2 mg/L 6-BA effective in *Justicia adhatoda* (Bhawna et al. 2017). However, in *Blepharis maderaspatensis*, which belongs to the same family, MS medium with NAA or 6-BA can only induce off-white, growth-inhibited callus (Drisya-das et al. 2014). In this study, we optimized the combination of 6-BA and NAA that is efficient for both callus and shoot induction. MS medium with 1 mg/L NAA and 2 mg/L 6-BA was found to be the best combination for callus induction in *H. difformis* (Fig. 2) whereas 0.1 mg/L NAA and 0.1 mg/L 6-BA was best for shoot induction (Fig. 3). Our results also revealed the pattern that low concentration of 6-BA and NAA is efficient for shoot induction, while high concentration of 6-BA and NAA induce the formation of callus, which is in consistent with the dose-dependent function of
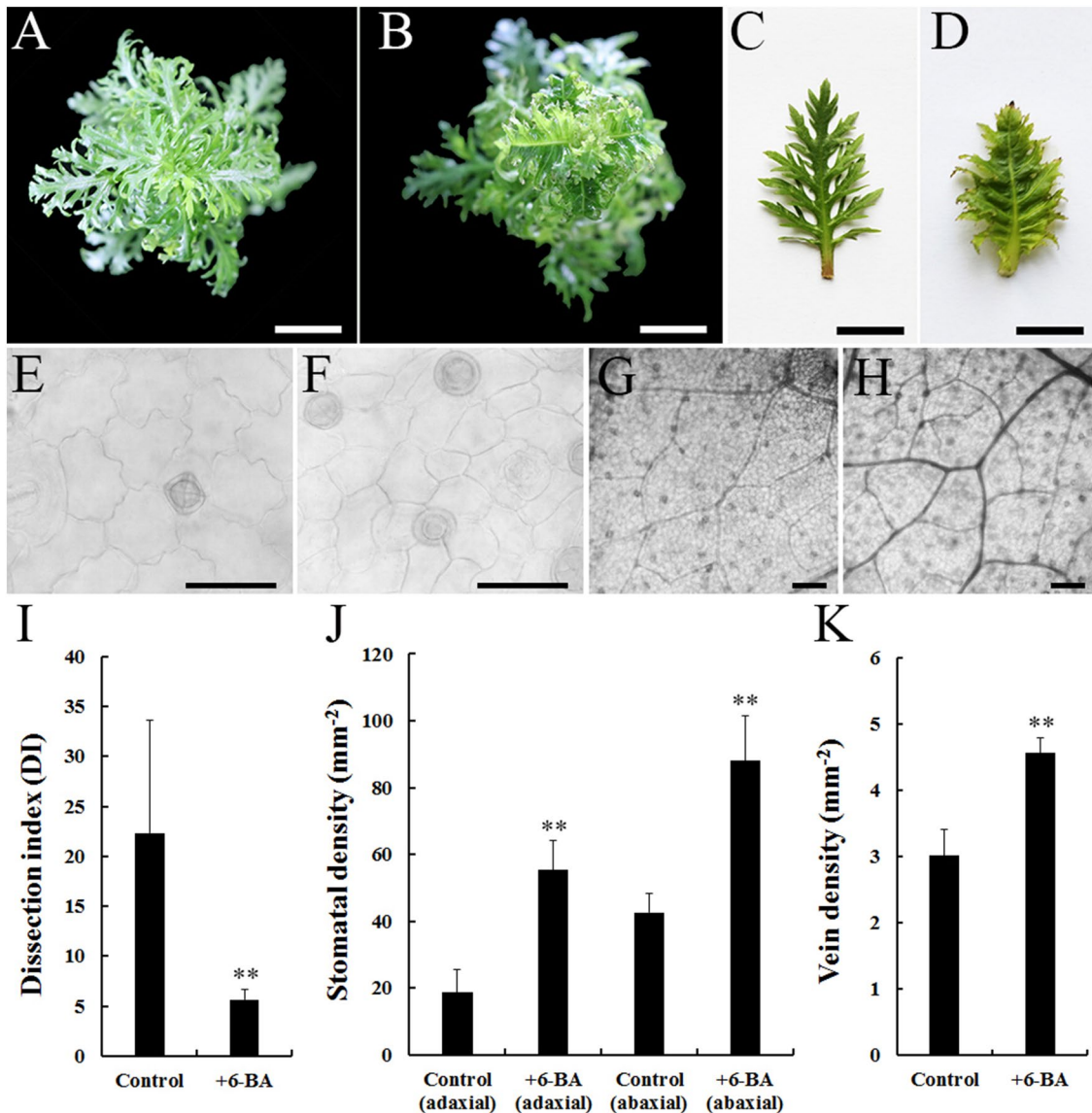
Fig. 7 Plant and leaf morphology upon control and CK treatment. **a** Top view of plants grown in 26 °C, 60% RH conditions. Note that plants grown in this condition and treated with 0.1% (*w/v*) ethanol was set as control. **b** Top view of plants treated with 10 μM 6-BA. **c** Leaf shape of control. **d** Leaf shape of plants treated with 10 μM 6-BA. **e** Epidermal cells of P6 leaves from control plants. **f** Epidermal cells of P6 leaves from plants treated with 10 μM 6-BA. **g** Minor veins of P6 leaves from control plants. **h** Minor veins of P6 leaves from plants treated with 10 μM 6-BA. **i** Dissection index (DI) of P6 leaves from plants with different treatments. Data are mean ± SD ($n=3$). **indicates significant difference to the control, $P<0.01$ (Student's t test). **j** Comparison of stomatal density: the number of stoma on both sides per unit area. Data are mean ± SD ($n=10$). ** indicates significant difference to the control, $P<0.01$ (Student's t test). **k** Comparison of minor vein density: vein length per unit area. Data are mean ± SD ($n=10$). ** indicates significant difference to the control, $P<0.01$ (Student's t test). Bars = 1 cm in (**a–d**). Bars = 100 μm in (**e–h**)

phytohormones in plant in vitro multiplication (Yang et al. 2018).

AS is another factor that has been previously shown to function in the transformation of many plant species including Arabidopsis (Sheikholeslam and Weeks 1987), soybean (Mariashibu et al. 2013), rice (Xi et al. 2018) and duckweeds (Yang et al. 2018). Our study revealed that AS is a crucial factor for high transformation efficiency of *H. difformis*.

The highest transformation efficiency was the treatment with 100 μM AS, with 15.6 ± 1% transformation efficiency (Fig. 4). Whiles pH of co-cultivation medium also plays a role in the transformation efficiency in some plant species (Ogaki et al. 2008), we found no significant difference in transformation using media of different pH (Fig. 4).

CKs are hormones which widely regulate cell division and function in many aspects of plant development (Shanks
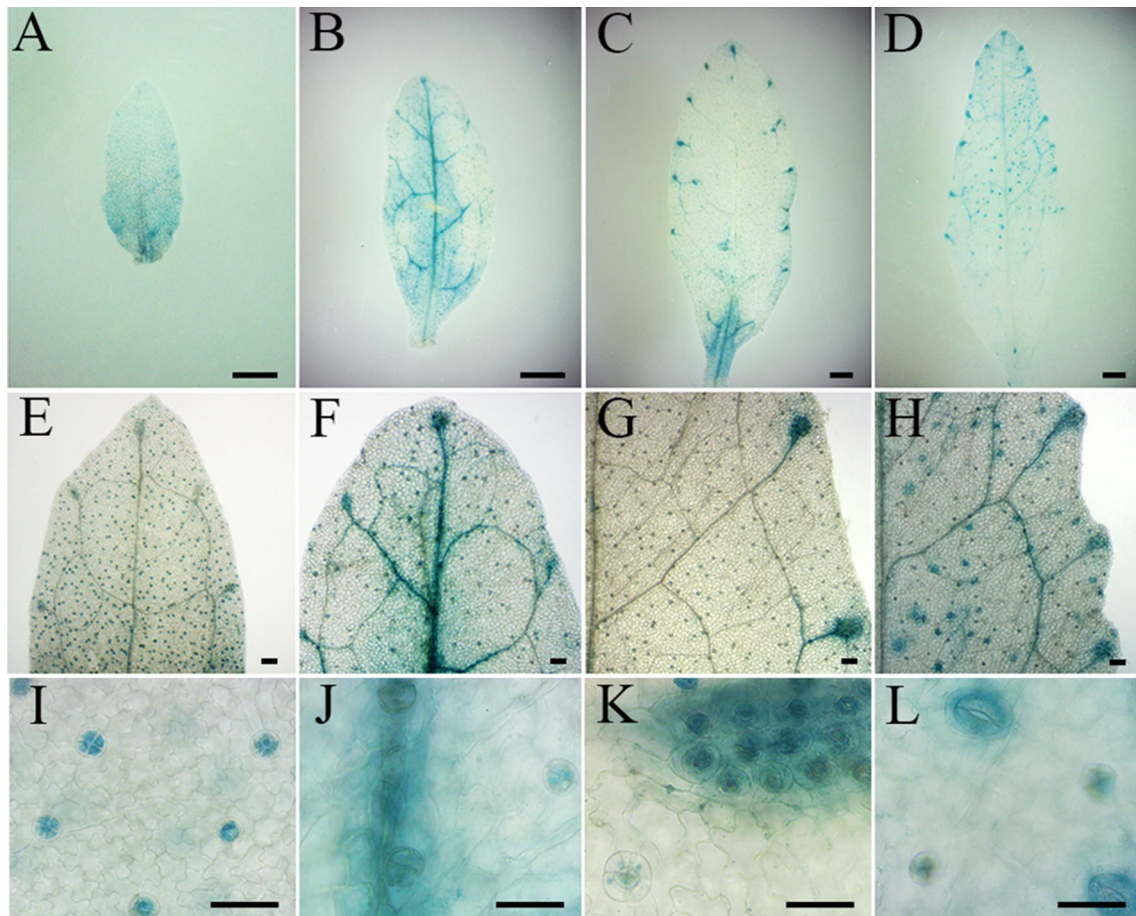
Fig. 8 Distribution of CK changes with leaf development of *H. difformis*. **a** Dispersed CK distribution in the whole leaf at the initial stage of leaf development and its magnification in (**e**, **i**). Note that CK occurred in most of the trichomes on the leaf surface. **b** GUS activity gradually restricted to the veins and hydathodes around leaf margins and its magnification in (**f**, **j**). Note that CK in the vascular bundles and trichomes on the leaf surface. **c** GUS activity sharply decreases and becomes more restricted to the hydathodes around leaf margins and its magnification in (**g**, **k**). Note that CK mostly occurs in the hydathodes around the leaf margin and trichomes. (**d**) GUS activity increases in the minor veins, meristems around the leaf margin and dispersed in the whole leaf and its magnification in (**h**, **l**). Note that CK mostly occurs in the developing guard cells and trichomes on the leaf surface. Bars =1 mm in (**a**–**d**). Bars =100 µm in (**e**–**l**).

et al. 2018; Takatsuka and Umeda 2019). Studies in the simple leaf plant Arabidopsis have shown that overexpression of *AtCKX* genes in transgenic plants resulted in decreased CK content, retarded shoot development, reduced leaf expansion, fewer but larger epidermal cells and decreased vein density in leaf (Werner et al. 2003). In the compound leaf plant tomato, heterologous expression of *AtCKX3* led to the production of simplified leaves that made only primary leaflets and smooth leaf margins. Transgenic plants had fewer, larger pavement cells and reduced stomatal density, whereas wild-type leaves treated with CK have increased number of pavement cells and increased numbers of stomata (Shani et al. 2010; Farber et al. 2016). However, little is known about the function of CK in aquatic plants or the role of CK in the heterophyllic transition. In the heterophyllous plant *R. aquatica*, CK levels decreased with high temperature and were higher in the compound than in the simple leaves.

Surprisingly, no leaf morphology change was observed following cytokinin treatment (Nakayama et al. 2014).

Our results indicate that CK mediates heterophylly in *H. difformis*, and specifically, that high levels of CK are required for the entire leaf phenotype typical of terrestrial conditions. First, we found that plants shoots producing highly dissected leaves induced by growth in high temperature and high humidity conditions (26 °C, 60% RH) have a low CK content, whereas shoots producing simple leaves induced by growth in either low temperature and high humidity conditions (20 °C, 60% RH) or high temperature and low humidity conditions (26 °C, 30% RH) have significantly higher content of CK (Fig. 6). We revealed that endogenous CK and leaf shape significantly changed with environmental factors, showing a similar response to temperature as *R.aquatica* (Nakayama et al. 2014) and it seems that the effect of humidity on heterophylly is more significant

than temperature. In addition, the negative correlation between reduced *HdCKX3* and CK levels under different humidity and temperatures, indicate that the difference of CK content may due to the expression of degradation-related genes. These results suggest that low content of CK may be necessary for compound leaf form in *H. difformis*.

This idea was further strengthened by the induction of entire leaves following treatment of plants with CK. We treated plants grown under high temperature and high humidity conditions (26 °C, 60% RH), with CK or its synthetic inhibitor, lovastatin whereas leaves produced by untreated plants resemble submerged leaves and have high DI, serrated epidermal cells, low stomatal density and vein density (Figs. 1, 7), plants treated with CK produced leaves with terrestrial features including low DI, polygonal epidermal cells, high stomatal and vein density (Figs. 1, 7). Plants treated with lovastatin had no obvious change of leaf shape perhaps because synthesis of CK is already inhibited under these conditions and lovastatin could not further limit its function.

It is known that leaf forms of heterophyllous plants are related to their function. Aquatic plants show the ''principle of reduction'': compared to characteristic features of terrestrial plants, distinct cell types, tissues, or organs are reduced or even missing in aquatic plants to withstand the destructive force of water and easily absorb its nutrients (Rascio et al. 1999). In heterophyllic plants, submerged leaves are thin, narrow and lack stomata, whereas terrestrial leaves are thicker, cutinized, expanded, with more lateral veins and stomata (Wells and Pigliucci 2000; Wanke 2011). One hypothesis is that dissected leaves can increase photosynthesis in submerged conditions because this type of leaf can incorporate more CO_2 (Baker-Brosh and Peet 1997). Another explanation is that leaf dissection could modulate leaf temperature (Helliker and Richter 2008).Our results provide evidence that CK levels determine multiple features of simple or complex leaves and play an important role of the regulation of heterophylly in *H. difformis*.

To answer the question of how CK might regulate heterophylly by altering leaf morphological characters, we used the *TCS::GUS* reporter construct to detect the dynamic distribution of CK during leaf development. Our results showed that the distribution of CK occurred in the trichomes at the initial stage, then in the midvein and secondary veins. As development progressed, GUS expression was high in the hydathodes around the leaf margin and then occurred in the guard cells of whole leaf (Fig. 8). These results suggested that CK functions in different stages of leaf development, firstly in trichomes, then in vein and hydathode formation, and finally in stomata development. The expression of *TCS::GUS* in trichomes at all developmental stages coincides with the view that cytokinin stimulates trichome development (Eckardt 2005; Gan et al. 2007). In future, it will be interesting to explore further the mechanism of CK in regulating heterophylly by comparing CK distribution in leaf development stages under terrestrial and submerged condition.

In conclusion, we developed an efficient protocol for stable transformation of the heterophyllous plant *H. difformis* by optimizing phytohormones for callus induction, shoot induction and identifying the crucial factor for transformation efficiency. In addition, we provided evidence for critical function of CK in the leaf development of *H. difformis* and revealed a role for CK in regulating heterophylly.

Author contributions HH planned and designed the research. GL, SH and JY performed experiments and data analysis. GL, SH, XZ, SK, EAS, HH contributed to the writing and editing of this article.

Compliance with ethical standards

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