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ORIGINAL ARTICLE

Cell shape can be uncoupled from formononetin induction in a novel cell line from *Callerya speciosa*

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Abstract

Key message **It is the first time that formononetin produced by cell culture and its accumulation was shown to be triggered by specific stress signalling linked jasmonate pathway.**

Abstract *Callerya speciosa*, an endangered traditional Chinese medicine plant, is intensively used in traditional folk medicine. To develop sustainable alternatives for the overexploitation of natural resources, a suspension cell line was created from *C. speciosa*. Ingredients of *C. speciosa*, for instance the isoflavone formononetin, are formed during a peculiar swelling response of the root, which is considered as a quality trait for commercial application. A cell strain with elongated cells was obtained by using synthetic cytokinin 6-benzylaminopurine (6-BA) and synthetic auxin picloram. Both, picloram and 6-BA, promote cell division, whereas picloram was shown to be crucial for the maintenance of axial cell expansion. We addressed the question, whether the loss of axiality observed in the maturating root is necessary and sufficient for the accumulation of formononetin. While we were able to mimic a loss of axiality for cell expansion, either by specific combinations of 6-BA and picloram, or by treatment with the anti-microtubular compound oryzalin, formononetin was not detectable. However, formononetin could be induced by the stress hormone methyl jasmonate (MeJA), as well as by the bacterial elicitor flagellin peptide (flg22), but not by a necrosis inducing protein. Combined the fact that none of these treatments induced the loss of axiality, we conclude that formononetin accumulates in response to basal defence and unrelated with cell swelling.

Keywords *Callerya speciosa* · Cell swelling · Picloram · Cell line · Formononetin · MeJA

Introduction

Callerya speciosa (Champion ex Bentham) Schot, meanwhile renamed as *Millettia speciosa*, belongs to the genus *Callerya* Endlicher, family Fabaceae (Flora of China, http://www.eflora.cn/foc/pdf/Callerya.pdf), and is found in the subtropical and tropical areas of Southern China and Vietnam. This plant is widely used for traditional folk medicine (Zong et al. 2009) with the first record traced back to the Qing Dynasty, a pharmaceutical monograph by He Ke-Jian (He 1711). In addition to *C. speciosa*, also other members of this genus, including *C. fordii, C. nitida*, and *C. oosperma* are used in Traditional Chinese Medicine (TCM) for different purposes (Zhang et al. 2008; Zong et al. 2009). In TCM, *C. speciosa* is used because it promotes blood circulation and relieves stasis. It is therefore used against arthritis, pertussis, haemoptysis, spermatorrhea, leucorrhoea, arthralgia, chronic hepatitis, traumatic injury, and others, and is also often used for tonifying soups and wines (Zong et al. 2009).

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Callerya speciosa is a liana, but for medical purposes, only the root is used, which undergoes a conspicuous swelling. The degree of swelling is considered a quality trait, and swollen roots achieve a higher price. However, this swelling process takes decades, and the extremely slow maturation of the root to the commercially valuable stage is a serious drawback for farmers cultivating this plant. The elongation of plant roots is shaped by strictly aligned cell divisions in the meristem, followed by cell elongation in the distal elongation zone. At the cellular level, the swelling process results from a loss of axiality, which is first leading to a lateral expansion of the cell, which is then followed by a tilted division axis leading to additional cell layers (Green 1980). Similar lateral root swellings have been observed in response to plant hormones. For instance, ethylene increases root width (Smalle and Van der Straeten 1997), and this effect of ethylene can be mitigated by blocking auxin transport, indicating that auxin signalling is relevant for root expansion (Strader et al. 2010). A second target for root swelling are the microtubules, because a transverse orientation of cortical microtubules is required to sustain the anisotropy of cell wall extensibility needed for elongation. In fact, treatment with anti-microtubular compounds, such as the polymerization inhibitor oryzalin, causes a characteristic transverse expansion of the root in *Arabidopsis* (Baskin et al. 2004).

As for most plants used in TCM, the active compounds in *C. speciosa* are still not completely elucidated, but several candidates with pharmacological activity have already been identified in both, stem and roots, of *C. speciosa*. These include formononetin (Wu et al. 2015), isoliquiritigenin, maackiain, pterocarpin, medicarpin, homopterocarpin, and oleanane-type triterpene saponins (Uchiyama et al. 2003; Zhang et al. 2008; Wang et al. 2011). Of additional interest are polysaccharides with anti-inflammatory and immunostimulating activity (Zheng 2009). The most interesting compound is the isoflavone formononetin, because it had been shown recently to stimulate estrogen-induced angiogenesis following vascular damage by activating actin contractility through the Rho-GTPase pathway (Li et al. 2015). The strong medical potency of *C. speciosa* has led to overexploitation of nearly all reachable wild resources. Since its slow growth habit makes cultivation economically unattractive, the plant has been moved to the verge of extinction.

Biotechnological production of the medically relevant compounds by plant cell fermentation would provide a sustainable alternative for relieving this rare species from disappearance (Kolewe et al. 2008). Since cultivated cells should harbour all the genetic information necessary to synthesize the secondary metabolites of interest, this approach has been explored for several medical plants such as *Catharanthus roseus* (Zhao et al. 2001), *Coptis japonica* (Yamada and Sato 1981), *Taxus spp*. (Frense 2007), or *Podophyllum spp*. (Arens et al. 1986). Although promising, this approach is often discouraged by absence of well proliferating cell cultures and low product yield (Wilson and Roberts 2012). Since secondary compounds are often accumulating in the context of stress responses, silent metabolic potencies can often be activated by elicitors, or by growth regulators involved in stress signalling (Yeoman and Yeoman 1996; Bringi et al. 2013). Despite considerable interest into plant cell fermentation of secondary compounds during the last decades of the past century, only few examples were developed to a stage that is commercially rewarding. The most successful example has been the fermentation of the anticancer compound paclitaxel using *Taxus chinensis* cell cultures in 75,000 L fermenters (Imseng et al. 2014).

According to the ethno-medical tradition, the efficacy of extracts increases with the swelling of the root. This is consistent with a recent transcriptomics study, where swollen roots were found to strongly upregulate genes involved in secondary metabolism in general, and genes of the phenylpropanoid pathway in particular (Xu et al. 2016). Also the biosynthesis of isoflavonoids (such as formononetin) is upregulated in swollen roots. This shifts the mechanisms underlying this root swelling into the focus of interest. If broken down to the cellular level, this phenomenon could be caused by three alternative scenarios:

1. The accumulation of the bioactive secondary compounds might be a consequence of lateral cell expansion (followed by lateral cell division). Such a link of metabolic potency with altered cellular differentiation had been shown for the patterned accumulation of anthocyanin in the mustard cotyledon, a classical model for pattern formation in plants (Nick et al. 1993). For this pattern, the vacuolar expansion of epidermal cells was found to be a precondition for the cellular competence for product accumulation induced by phytochrome A (Steinitz and Bergfeld 1977).
2. Alternatively, the later cell expansion (followed by lateral cell division) might be a downstream effect of product accumulation. For instance, a water-soluble compound that accumulates in the vacuole would decrease osmotic potential, which would activate mechanosensitive channels and lead to a compensatory response of the vacuole. Such regulatory networks are central for the adaptation to salt stress (reviewed in Ismail et al. 2014).
3. As a third possibility, cells might respond to a triggering signal by arresting their cell cycle and undergoing a transition into cell expansion and differentiation. Here, the two phenomena (swelling and product accumulation) would correlate as consequences of a common cause, but without a direct causal interrelation.

A peculiar feature of *C. speciosa* is the fact that the root is not simply elongation but also swelling unevenly

in lateral direction. This implies a loss of cell axiality, a feature that is typically linked with a progressive loss of cell wall anisotropy (Nick 2012). For geometrical reasons, the lateral component of expansion stress is twice that of the longitudinal component, such that a loss of elongation in an expanding tissue will lead to lateral expansion as default state (Green 1980). This lateral expansion would then later be followed by cell division in the tilted axis, resulting in additional cell layers. Since the texture of the secondary cell wall depends on a directional movement of cellulose-synthase complexes in the cell membrane that is guided by cortical microtubules subtending the cytoplasmic face of the membrane (Lloyd and Chan 2008), the root swelling might be linked with reorganization of cortical microtubules. In fact, a loss of microtubule order was shown to underlie root swelling in a mutant of Arabidopsis (Bannigan et al. 2006). Also, the tilted division axis, often observed as long-term consequence of altered expansion axiality, depends on microtubular organization, because it is guided by a plant-specific array, the preprophase band (Nick 2012).

To get insight into the cellular mechanisms responsible for root swelling and product accumulation, a study in the whole-plant context is experimentally very difficult, or even impossible for *C. speciosa*, because the roots grow and swell extremely slowly. We therefore generated a suspension cell line from this species, where expansion axiality can be induced by a specific treatment with picloram (mimicking auxin activity) and 6-benzylaminopurine (mimicking cytokinin activity). This experimental system made it possible to ask the following questions: (1) what is the relation between the isodiametric cell expansion and the accumulation of secondary metabolites? (2) Is the swelling response linked with microtubules? To address these questions, we established a novel cell suspension culture from *C. speciosa* and optimized the cell growth by changing the ratio and concentration of growth regulators. As readout for the accumulation of bioactive secondary metabolites, we measured formononetin, as most valuable compound of *C. speciosa* by high-performance liquid chromatography (HPLC). To detect a potential role of microtubules in the swelling response, we conducted morphometry after treatment with oryzalin, a compound suppressing the integration of tubulin dimers into microtubules, and taxol, a compound suppressing the disassembly of microtubules. We show that formononetin accumulation can be triggered by exogenous methyl-jasmonate, whereas flg22, an activator of basal defence is ineffective. We further show that the swelling response can be modulated by pharmacological manipulation of microtubules. We also can define conditions to uncouple cell swelling and product accumulation providing evidence for two separate, but parallel pathways.

Materials and methods

Cell cultures

Callus of *C. speciosa* was generated from tender stem (Huang et al. 2008) and maintained on 0.27% gellan gum (Sigma-Aldrich) containing 4.43 g.L^{-1} Murashige and Skoog basal medium with vitamins (Phytotechnology Laboratories), 30 g.L^{-1} sucrose, 1 mg L^{-1} 6-benzylaminopurine (6-BA) (Sigma-Aldrich), 0.5 mg L^{-1} picloram (Sigma-Aldrich), pH 5.9. Calli were sub-cultured monthly. Three-week-old calli were transferred into liquid medium (same medium as for calli but omitting gellan gum) to create suspension cell cultures. Suspension culture and calli were both kept in dark, 25 °C. Suspension cells were sub-cultured every 10 days, inoculating 5 mL of stationary cells into 30 mL of fresh medium in 100 mL Erlenmeyer flasks on an orbital shaker (Kuhner Shaker, ISF4-X, Germany, Φ=25 mm) at 120 rpm.

Effect of growth regulators and cytoskeletal drugs on cell growth

To monitor the cellular response to 6-BA, picloram was fixed to 0.5 mg L^{-1} and 0, 0.25, 0.5, 1, 2, 4, or 8 mg L^{-1} of 6-BA were added at sub-cultivation. The reason, why picloram was fixed to 0.5 mg L^{-1} was that form this concentrations the response of cell size to picloram was saturated; to assess the response to picloram, 0, 0.25, 0.5, 1, 2, 4, 8 mg L^{-1} of picloram were added at sub-cultivation, while 6-BA was fixed to 1 mg L^{-1}. Again, this concentration ensured a saturating effect of 6-BA on the suppression of cell size. Cell growth was quantified by measuring packed cell volume (PCV) at 10 days after sub-cultivation (Chang et al. 2011). Since the relationship between PCV and fresh weight was found to be linear with: $Y = 1.05X$, $R^2 = 0.9858$, with X = PCV, Y = fresh weight, we used PCV as convenient readout for culture growth.

To quantify the response to cytoskeletal drugs (sterilized by filtration), taxol was added to the medium at sub-cultivation in a concentration of 0, 0.25, 0.5, 1, 2, 4, or 8 µM. Oryzalin, at 0, 0.25, 0.5, 1, 2, 4, 6, 8 µM, respectively, was added in a parallel set of experiments at sub-cultivation. For the taxol treatment, the medium was supplemented with 0.5 g L^{-1} 6-BA and without picloram, whereas for the oryzalin treatment, the medium was containing 0.5 mg L^{-1} picloram only.

Data represent mean values and standard errors from at least three independent experimental series.

Determination of cell morphology, cell viability and mitotic indices

Cell viability (Chang and Nick 2011) and mitotic index (Maisch and Nick 2007) was quantified at days 0, 2, 4, 6, 8, 10 after sub-cultivation, respectively. For each data point, 1500 cells from 3 independent experiments were scored.

At day 10 after sub-cultivation, when the culture had reached stationary phase (Fig.S1), the cells were imaged by a digital image acquisition system with a cooled digital CCD camera (AxioCamMRm; Zeiss) (Axiovision) under an AxioObserver Z1 (Zeiss, Jena, Germany) using 10× or 20× objectives. Cell perimeter and length of the long cell axis were measured by the Image J software (https://imagej.nih.gov/ij/). Data represent mean values and standard errors from more than 300 individual cells collected from 3 independent experimental series.

Elicitors and treatment

MeJA was purchased from Sigma-Aldrich (Shanghai, China). Flg22 (Peptide sequence: QRLSTGSRINSAKDDAAGLQIA) was synthesized by Sangon Biotech (Shanghai, China). NLP_{Fo} was expressed and purified by Sangon Biotech (Shanghai, China) based on the gene sequence for NLP of *Fusarium oxysporum* (GenBank accession AF036580).

MeJA (1 µM, 5 µM, 10 µM and 20 µM), flg22 (250, 500, 750 and 1000 nM), and NLP_{Fo} (50, 100, 200, and 500 µg L^{-1}) were added into the cell suspension at day 8 after sub-cultivation in MS medium containing 1 mg L^{-1} 6-BA and 0.5 mg L^{-1} picloram, pH 5.9. After further 20 h of cultivation, cells were harvested by filtration for formononetin extraction.

Extraction of the active metabolite formononetin

Aliquots (10 mL) of the suspension cells were harvested 20 h after elicitation (taking place at day 8 after sub-cultivation) by centrifugation (2744×*g*, 5 min). After removing the supernatant, 1.0 g fresh weight of cells were mixed with 10 mL methanol and extracted by an ultrasonic processor (VC130, Sonic & Materials Inc., USA) for 45 min, filtered and then transferred to a 10-ml volumetric flask. The filtrates were filtered through a 0.22 µm microporous membrane before analysis by HPLC. To determine the formononetin content in the medium, the supernatant was collected after spinning down the cells, and filtered for HPLC analysis. Three replicates of each sample were prepared and analysed.

The separation by HPLC was performed on an Agilent infinity 1260 (Agilent Technologies) device equipped with a ZORBAX Eclipse XDB-C18 column (250 mm × 4.6 mm, 5 µm). The mobile phase was a mixture of methanol with 0.2% (v/v) phosphoric acid at 65:35 (v/v), administered at a flow rate of 1 ml min^{-1}. For detection, a wavelength of 248 nm was used, column temperature was kept at 30 °C.

The contents of formononetin were calculated using a linear regression equation. Methanol was added to a formononetin standard powder (≥ 98%, Nanjing Spring & Autumn Biological Engineering Co., Ltd., China) to yield a concentration of 0.176 mg mL^{-1}. This formononetin standard solution was diluted to five calibration concentrations and measured under the HPLC conditions described above. The HPLC quantification was validated by linear regression analysis, as well as tests for recovery, stability, precision, and reproducibility. The results of the calibration followed a strictly linear relationship: $Y = 104183X + 5.0231$, $R^2 = 1$ with X formononetin concentration, and Y peak area.

Statistical analysis

Significantly different at the 95% confidence level was analysed by SPSS 10.0.

Results

Establishment of a suspension cell culture for *C. speciosa*

For medical application, typically, well swollen roots of *C. speciosa* are excavated from the wild (Fig. 1a). The bigger the size, the higher the price, leading to over exploitation of this species that in consequence becomes rarer and rarer. Upon cultivation of this plant, the extremely slow growth becomes evident, after 6 months, the roots have extended to only ~ 0.5 cm in diameter (Fig. 1b). As visible from cross sections of roots prior (Fig. 1f) and subsequent to (Fig. 1g) swelling, the axiality of the cells in the primary root cortex is tilted (Fig. 1f, insets). Prior to swelling, cortical cells appear isodiametric in the cross section, indicative of a cell axis aligned with the long axis of the root (Fig. 1g, inset). In contrast, they are clearly oriented in radial direction, when swelling has initiated (Fig. 1g, inset). This tilted expansion is followed by radial divisions, giving rise to parenchymatic rays, sclerenchymatic tissue and vascular bundles. These differentiated tissues exhibit a pattern of concentric rings indicative of a rhythmic activity of cambial cells.

To develop alternatives, a callus was induced by growth regulators in surface-sterilized tender stems of *C. speciosa* (Fig. 1c), and this callus was later transferred to liquid medium to establish a cell line. The resulting suspension cells were of elongated shape (Fig. 1d), and divided parallel with this pronounced axiality giving rise to pluricellular files (Fig. 1e), thus mimicking the situation in the young root cortex, prior to radial swelling.

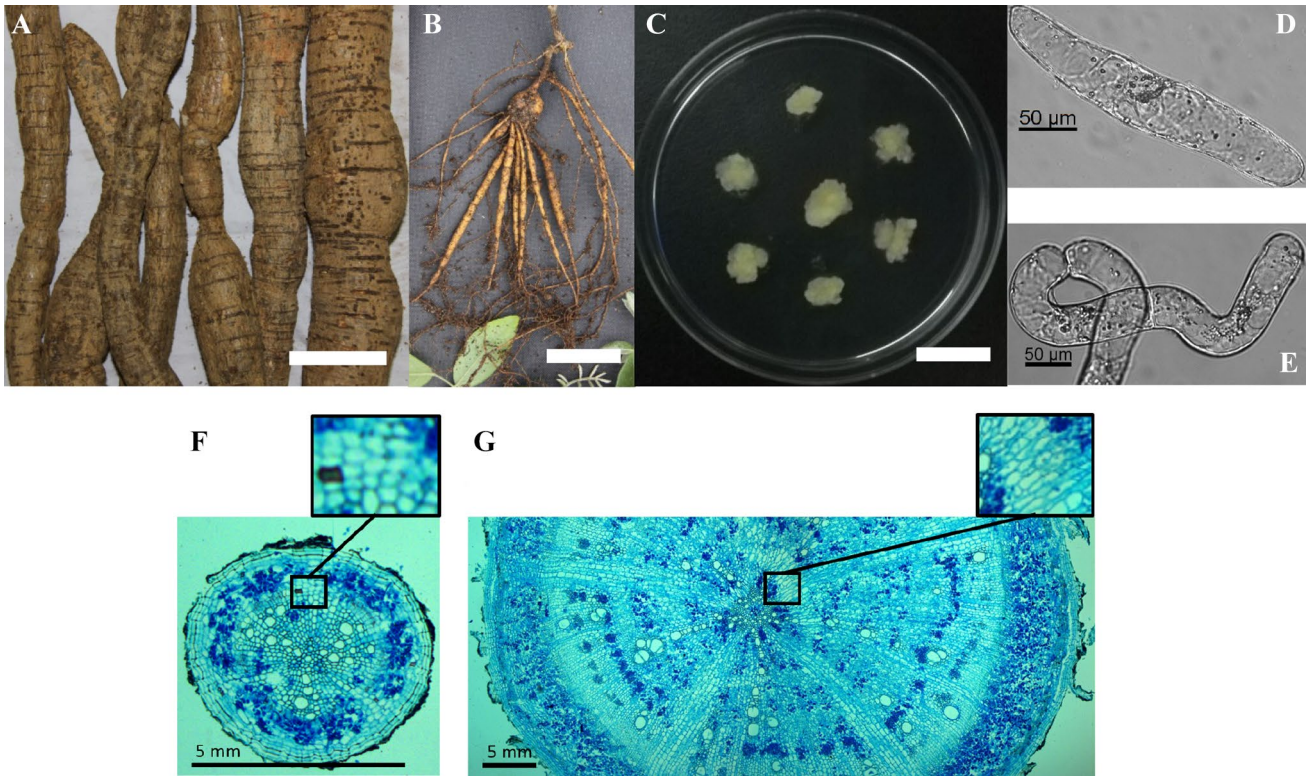

Fig. 1 Morphology, anatomical aspects of roots and cells of *C. speciosa*. **a** Perennial roots of *C. speciosa* from plants collected in the wild as typically traded for medicinal use. Bar = 5 cm; **b** initiation of root swelling in a plant cultivated for 6 months. Bar = 3 cm; **c** calli induced from slender stem. Bar = 2 cm; **d, e** representative suspension cultured cells derived from calli, showing the pronounced elongation of single cells (**d**) and the strict axiality of pluricellular cell files (**e**). **f, g** Transverse sections of a young root prior to swelling (**f**), and a root that has undergone swelling over three years (**g**). The insets show the region of the primary root cortex, where cell axiality has tilted by 90°, such that the cortical cells expand in transverse direction, which is later followed by radial divisions of the intermittent cambium. The diameter of F = 0.65 mm and G = 8.42 mm. The sections were stained with 0.1% fast green and observed under light microscopy with 5× objective

Both picloram and 6-BA can promote cell division

The callus has been successfully induced from tender stems by screening different growth regulators according to Huang et al. (2008). With the combination of picloram and 6-BA, callus can be easily induced and the callus can proliferate quickly. The cell line underwent active mitosis and cell mortality kept low (Fig. S1). It took about 10 days for a mitotic cycle and cells to reach interphase again where the cell number did not increase anymore. Therefore, packed cell volume (PCV) was used as readout for cell culture growth, whereas cell perimeter and axis length were used to indicate cell morphology change.

To assess the cellular response to picloram and 6-BA, different concentrations of these growth regulators were added in during subculture, while the concentration of the other regulator was kept constant. When fixing concentration of picloram to 0.5 mg L^{-1} (which ensured that the effect on cell size was saturated) while increasing the concentration of 6-BA from 0 to 8 mg L^{-1}, both packed cell volume and cell perimeter had decreased (Fig. 2a), if scored 10 days later. However, this decrease was different in amplitude: while packed cell volume gradually decreased down to around 40% compared to the value in the absence of 6-BA, cell perimeter dropped much more sharply, down to a third of the initial value, when the 6-BA concentration was raised to 0.5 mg L^{-1} (where packed cell volume still had not significantly changed). For higher concentrations of 6-BA, the perimeter saturated at this small value. In other words: especially concentrations of 6-BA up to 0.5 mg L^{-1} generated cells that were much smaller, while packed cell volume was only mildly affected, meaning that the reduction in cell size must be compensated by a corresponding increase in cell number. These results indicate that low concentrations of 6-BA stimulate cell division, while cell expansion was inhibited.

The function of picloram was assessed in a similar way by fixing the concentration of 6-BA at 1 mg L^{-1} (which ensured that the effect on cell size was saturated) and progressively increasing the concentration of picloram. Here, the packed

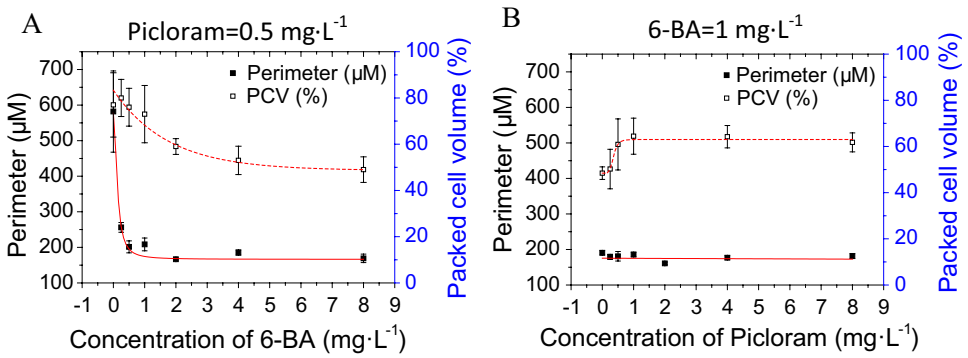

Fig. 2 Effect of picloram and 6-BA on cell growth. Response of packed cell volume and cell perimeter for, **a** a fixed concentration of picloram (0.5 mg L^{-1}) over rising concentrations of 6-BA, or, **b** a fixed concentration of 6-BA (1 mg L^{-1}) over rising concentrations of picloram. Data represent mean values and standard errors from three biological replicates

cell volume increased by around 20%, when picloram concentration was raised to 1 mg L^{-1}, and then remained at this level for higher concentrations of picloram. In contrast, the perimeter remained constant throughout (Fig. 2b). The constant cell size for increasing concentrations of picloram in concert with the increase of packed cell volume is indicative of a rise in cell number. Thus, low concentrations of picloram can promote cell division, too. However, compared to the effect of 6-BA, this stimulation is of minor amplitude.

6-BA inactivates expansion axiality

The root swelling indicates that expanding cells progressively lose their axiality, leading to the question, whether this could be mimicked in cell culture by growth regulators. We therefore plotted the ratio of cell perimeter over the long axis of the cell. In a perfectly round cell, this ratio would be equal to π (3.14), while in a very long and thin cell, this ratio would approach 2. In fact, drastic changes of cell axiality could be observed: when the cells were cultivated in the absence of 6-BA at a low concentration of picloram (0.5 mg L^{-1}) to ensure sufficient cell expansion (Fig. 2b), the ratio perimeter/long axis was 2.2 (Fig. 3a) indicative of elongated cells. By increasing the concentration of 6-BA, the ratio rose rapidly to almost 3, indicative of cells that were almost completely round. Conversely, cells that were cultivated in the absence of picloram, but in presence of 1 mg L^{-1} 6-BA were almost round with a ratio of more than 2.7 (Fig. 3b). By raising the concentration of picloram, they became more elongate, but only slightly. Thus, 6-BA is not only stimulating cell division (Fig. 2a), but also can cause a massive loss of axiality (Fig. 3b). In contrast, picloram supports and slightly stimulates axiality (Fig. 3a). In summary, the cellular aspects of root swelling can be mimicked in the cell culture by modulation of growth regulators.

Pharmacological manipulation of microtubules modulates cell axiality

To test whether microtubule cytoskeleton are involved in cell axiality, different concentrations of the microtubule-stabilizing compound taxol were added during subculture in the presence of 0.5 mg L^{-1} of 6-BA alone. This concentration was chosen, because under these conditions, cells were

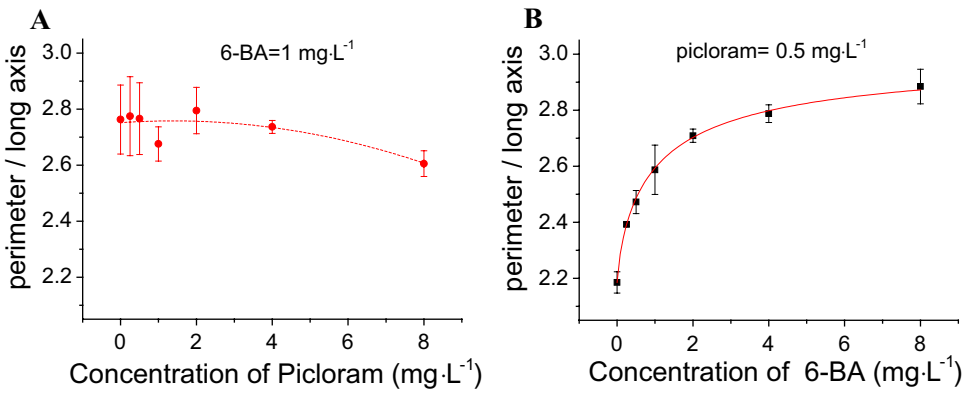

Fig. 3 Effect of picloram and 6-BA on cell shape. **a** Ratio of perimeter over the long cell axis as measure for cell shape, a fixed concentration of picloram (0.5 mg L^{-1}) over rising concentrations of 6-BA, or, **b** a fixed concentration of 6-BA (1 mg L^{-1}) over rising concentrations of picloram. A value of 3.14 (π) would correspond to a completely spherical cell, a value of 2 would correspond to a perfect filament without cross extension. Data represent mean values and standard errors from three biological replicates ($n \geq 300$)

round with almost complete loss of axiality (ratio perimeter/long axis almost 2.9), what allowed to see a potential stabilization of expansion axiality. Addition of taxol increased cell perimeter and packed cell volume slightly (Fig. 4a), while the ratio of perimeter/long axis decreased drastically to a saturation value of around 2.5 reached from less than 1 µM of taxol (Fig. 4b). Thus, stabilization of microtubules by taxol can significantly stabilize cell axiality against the influence of 6-BA.

When the cells were challenged by oryzalin, a compound sequestering tubulin dimers from integration into microtubules, such that microtubules are eliminated due to their innate turnover, cell perimeter and packed cell volume both decreased (Fig. 5a), with the decrease of perimeter being more pronounced. When the cells were cultivated only in presence of 0.5 mg L^{-1} picloram, cells were elongate, with a ratio of perimeter/long axis close to 2 (Fig. 5b). This setting was therefore appropriate to detect a potential destabilization of expansion axiality.

This response was accompanied by a dramatic increase in the ratio of perimeter/long axis following a sigmoidal dose–response curve. The threshold for this increase was at ~2 µM oryzalin and reached a plateau at ~3µM. Here, a ratio of 3.1 was established, meaning that the cells formed a perfect sphere, such that the ratio perimeter/long axis approximated π (Fig. 5b). This suggests that microtubules are necessary for maintenance of cell axiality.

Since stabilization of microtubules through taxol promotes the stability of cell axiality against 6-BA, while elimination of microtubules through oryzalin eliminates cell axiality despite the presence of picloram, these data demonstrate that microtubules are necessary and sufficient for cell axiality.

Formononetin eliciting and determination in the *C. speciosa* cell line

Formononetin belongs to the main active ingredients in the root of *C. speciosa*, and is considered as important value-giving compound (Wu et al. 2015), since it can activate estrogen-dependent actin remodelling and contractility, promoting angiogenesis following vascular damage and wound healing (Li et al. 2015). The correlation between root swelling, medical value, and formononetin content led to the question, whether the modulation of cell axiality by 6-BA and picloram would result in corresponding changes of formononetin content. Alternatively, activation of stress signalling by MeJA, activation of basal defence by

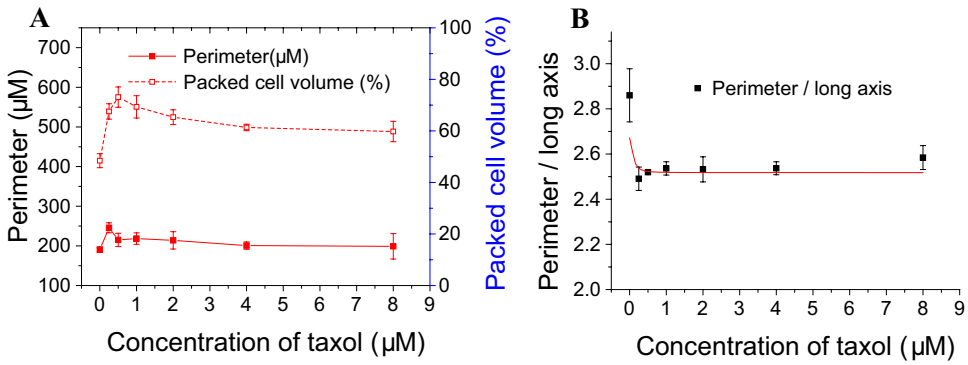

Fig. 4 Dose response of the microtubule stabilizer taxol. **a** Cell perimeter and packed cell volume over rising concentrations of taxol. **b** Ratio of perimeter/long axis over rising concentrations of taxol. The concentration of 6-BA was constant (0.5 mg L^{-1}), while picloram was omitted from the assay. Data represent mean values and standard errors from three biological replicates

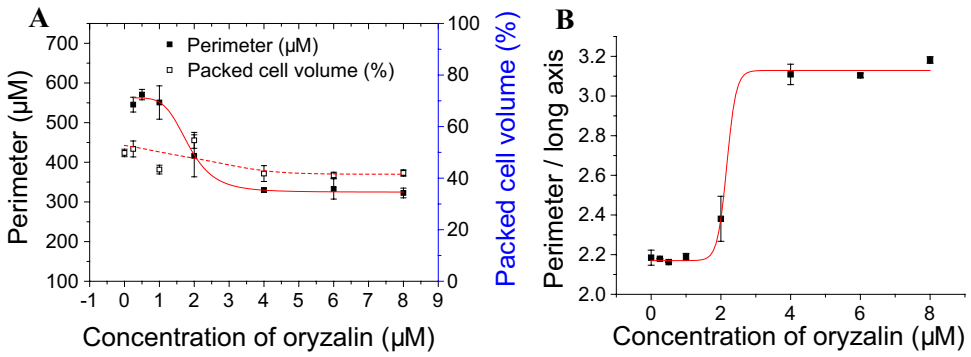

Fig. 5 Dose response of the microtubule eliminating agent oryzalin on cell shape. **a** Cell perimeter and packed cell volume over rising concentrations of taxol, **b** ratio of perimeter/long axis over rising concentrations of taxol. The concentration of picloram was constant (0.5 mg L^{-1}), while 6-BA was omitted from the assay. Data represent mean values and standard errors from three biological replicates

the bacterial elicitor flg22, or activation of cellular necrosis by the fungal toxin NLP_{Fo} were investigated for their effect on formononetin synthesis. The content of formononetin was measured through HPLC, by recording chromatograms and peak areas of formononetin were recorded and using a calibration curve based on a commercial standard (for details refer to the method section).

Cells were harvested at 9 days after sub-cultivation for the quantification of formononetin. At this time point, the culture had reached its stationary phase, as seen from a maximal value for packed cell volume (PCV). During our preparatory work, we had seen a very close and linear relationship between PCV and fresh weight, such that it was feasible to use PCV as convenient readout for fresh weight.

Formononetin was not detected under control conditions (1 mg L^{-1} 6-BA and 0.5 mg L^{-1} picloram), nor under other combinations of the two growth regulators (Table S1), even for such combinations that would induce a loss of axiality. Addition of the fungal toxin NLP_{fo} did likewise (Table S2). However, formononetin can be elicited by MeJA and, though to a much lower extent, by flg22. As shown in Fig. 6a, the content of formononetin increased in a dose-dependent manner with 0.015 mg g^{-1} fw in response to 1 µM MeJA till 0.113 mg g^{-1} fw in response to 5 µM MeJA, i.e. by 7.5 times. Even after 20 h of treatment with MeJA, no browning of cells was observed, speaking against the possibility that MeJA acts via activation of cell death. This level represented saturation, because when MeJA was further raised to 10 µM and 20 µM, this did not significantly promote the accumulation of formononetin. Also the bacterial elicitor flg22, an activator of basal immunity (Felix et al. 1999), was able to induce the accumulation of formononetin. However, here only about 0.011 mg g^{-1} fw was reached, i.e. a level corresponding to that seen for 1 µM MeJA. Thus, while combinations of 6-BA and picloram that can induce a loss of axiality, are not efficient in activating formononetin, MeJA which does not cause any obvious loss of cell axiality (Fig. S2), can elicit the accumulation of this compound (as well as the bacterial elicitor flg22, which is much weaker, though).

Discussion

Plants can produce about 10^6 secondary metabolites, and many of them have been exploited for medical use (Saito and Matsuda 2010). Biotechnological production of these metabolites via plant cell fermentation represents a sustainable strategy to the conventional collection of wild plants, avoiding over consumption of natural resource, and, thus, sparing rare plants from extinction. Here, we report a newly established cell culture from the endangered traditional Chinese medicine plant *C. speciosa*, where medical efficacy is correlated with a peculiar swelling of the root, which develops extremely slowly, rendering the cultivation of this rare and precious plant economically unfeasible. This suspension cell culture is characterized by a synchronized growth cycle, fast growth rate, and pronounced axiality of expansion and division. The accumulation of biomass (monitored by packed cell volume at interphase), and cellular morphology can be manipulated by changing the ratio of growth regulators 6-BA and picloram. By specific combinations of growth regulators, but also by treatment with low concentrations of

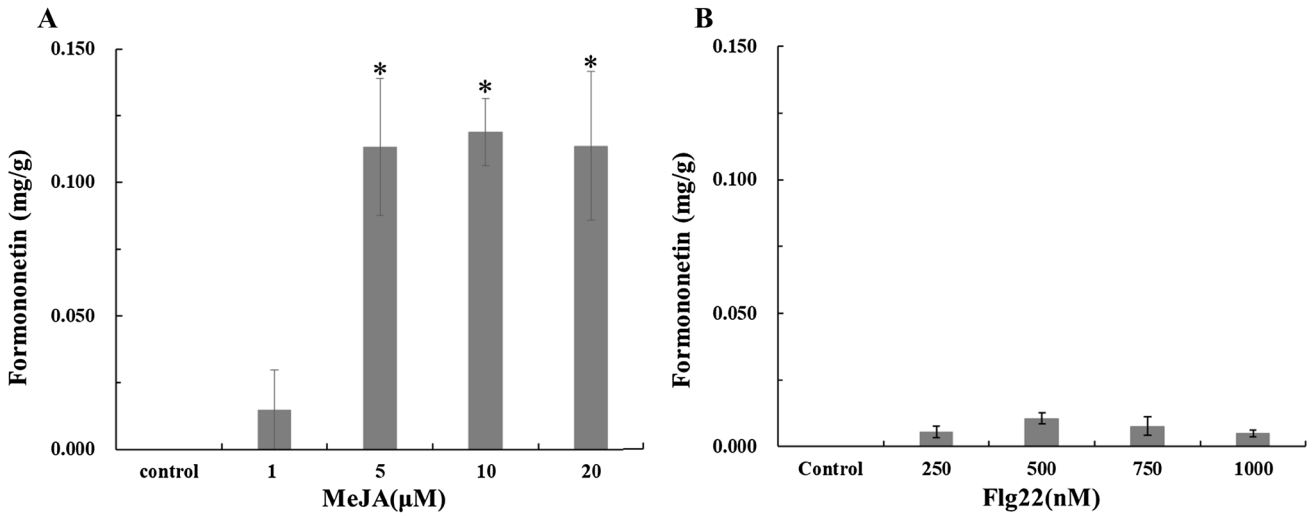

Fig. 6 Quantification of formononetin in suspension culture cells of *C. speciosa* after elicitation with MeJA (**a**) and flg22 (**b**). Cells were induced at day 8 after sub-cultivation with the indicated concentrations of MeJA for 20 h, and then the content of formononetin was determined by HPLC. Data represent mean values and standard errors from three biological replicates. *Significantly different from the untreated control at the 95% confidence level

the anti-microtubular herbicide oryzalin, we succeeded to induce a loss of cellular axiality, mimicking the situation in the root parenchyma of swelling root. This allowed dissection of the causal relationship between cellular morphology and accumulation of formononetin. We find that the loss of cellular axiality is not sufficient to induce formononetin. Instead, we can induce formononetin by treatment with MeJA, as well as by activation of basal immunity, although this treatment does not interfere with cellular axiality. From these data we can draw conclusions on the mode of action of the artificial growth regulator picloram with respect to specific aspects of auxin signalling. While the accumulation of interesting medical compounds in plant cell cultures has been described for a couple of cases, to our knowledge, it is the first time, that the link between cellular morphogenesis and product accumulation has been addressed in a plant cell culture. So far, all biotechnological approaches in plant cell cultures have treated the cells as more or less amorphous "biomass". By looking at the cellular details of product accumulation, we can show that the activation of morphogenetic changes (loss of cell axiality) as it occurs in the swelling root, is not the cause, but a byproduct of the differentiation event that releases the metabolic potency to generate the compound of interest, the isoflavone formononetin.

The herbicide picloram mimics auxin activity with respect to cell axiality and promotion of cell division

Picloram is a derivative of picolinic acid and has been primarily used as herbicide for woody plants and broadleaf weeds. Similar to other herbicides with auxinic activity, picloram was found to be a useful tool for plant tissue culture, since it can enhance the growth of calli, improve rooting in vegetative propagation of shoot cuttings, and activate somatic embryogenesis, even in otherwise recalcitrant systems, such as *Phyla nodiflora* (Eisinger and Morré 1971; Ahmed et al. 2011), yew (Furmanowa et al. 1997), palm (Valverde et al. 1987), and cassava (Groll et al. 2001). The active concentration within the cell is not known neither, and we have therefore used dose–response curves for both picloram and BA. The concentrations used in our study are comparable to those used in the field, for instance, our maximum concentration of picloram (8 mg L^{-1}, corresponding to 125 µM) is almost identical to that used to test the role of AFB5 as potential receptor for picloram in Arabidopsis (100 µM, Walsh et al. 2006). We also followed viability in all our experiments and did not see significant increases by the treatment with picloram or BA in the concentrations used. While the effects of picloram are indicative for an activation of auxin signalling, the mode of action remains unknown. A mutation in the auxin receptor homologue AFB5 conferred resistance to picolinate compounds, while the mutant remained sensitive to 2, 4-D and to IAA (Walsh et al. 2006) indicative of several receptors with different patterns of ligand affinity. A similar conclusion had been obtained, when axial cell expansion and cell proliferation were phenotyped in response to different concentrations of 2, 4-D versus NAA (Campanoni and Nick 2005). Here, cell proliferation was more efficiently triggered by 2,4-D and found to depend on G-protein activity, while axial cell expansion was activated preferentially by NAA, and was independent of G-proteins. In our newly established suspension cell culture, picloram produced only a slight promotion of cell division, but was very efficient in sustaining cell axiality (Figs. 2, 3). In other words, picloram acted here rather like NAA, not like 2, 4-D. This would also explain the finding by Walsh et al. (2006) that the *afb5* mutant of Arabidopsis was resistant to picolinates, but remained sensitive to 2, 4-D. The most straightforward interpretation would be that picloram activates the NAA-affinity auxin receptor. A testable implication of this hypothesis would be that cell axiality in the presence of picloram should persist treatment with pertussis toxin, an inhibitor of G-protein activity (because the signal activated by binding of picloram to NAA-affinity auxin receptor should be independent of G-protein activation).

Cell axiality in *C. speciosa* is under control of microtubules and auxin signalling

In the primary state, root growth relies upon cell division taking place in a very narrow meristematic zone at the root tip, and cell expansion (taking place in the distal expansion zone just proximal to the meristem). For older roots, such as those specimens of *C. speciosa* that are commercially relevant, secondary growth is more relevant. However, this secondary growth is brought about by the cambial activity in the pericycle. Inspection of the root anatomy (Fig. 1f, g) shows a peculiar tilt of cell axiality in the primary root parenchyma, just adjacent to the endodermis. While these cells show an isodiametric cross section in the young, slender root (meaning that these cells are aligned parallel to the root axis), their axis is clearly radial in the swollen root (meaning that these cells have tilted their axis by 90°). This tilted axis of parenchymatic expansion is accompanied by radial files of pith rays towards the periphery, indicative of a mechanism, where the cells first switch their expansion axis and subsequently align their division axis in the same direction resulting in lateral rather longitudinal expansion. Orientation and organization of microtubules play a key role for directional cell expansion (Wasteneys and Galway 2003; Bianco and Kepinski 2011). For instance, disassembly of microtubules by oryzalin can not only inhibit elongation of the seminal root in *Arabidopsis thaliana*, but also induce lateral swelling of the root zone, leading to a peculiar club-shaped appearance (Baskin et al. 2004). Although the stimulation of cell

expansion by auxin is not exclusively caused via microtubule orientation (see, for instance, Baskin 2015; Nick et al. 1991), microtubule orientation can determine the axiality of cell expansion is well established by classical studies (Shibaoka 1994). The pronounced cell elongation in the suspension culture of *C. speciosa* was therefore predicted to be strongly reduced by treatment with oryzalin. In fact, we observed that the ratio between perimeter and long axis was almost equal to π, indicative of a complete loss of expansion axiality (Fig. 5). On the other hand, the microtubule stabilizing compound taxol could strongly reduce this ratio in cells that were cultivated in presence of 0.5 mg.mL^{-1} 6-BA (Fig. 4). These cells that would be almost completely spherical in response to 6-BA, were able to sustain their axiality at least partially, if microtubules were stabilized. In other words: microtubules are necessary (Fig. 5) and (to a large extent, but not completely) sufficient to sustain the axiality of cell expansion. This does not exclude that other players, in addition to microtubules, support axiality.

Formononetin accumulation is uncoupled from expansion axiality, but linked with basal immunity

Root swelling is linked with activation of genes responsible for secondary metabolism including the general phenylpropanoid pathway and, specifically, the activation of isoflavones such as formononetine (Xu et al. 2016). This supports the traditional view on medical efficacy in *C. speciosa*, which is reflected in a much higher price of the aged, swollen roots. On the cellular level, root swelling is linked with the loss of longitudinal axiality in the cells of the primary cortex (Fig. 1f, g), leading to the question, whether the loss of longitudinal axiality might be a prerequisite for the accumulation of medically active compounds. In this study, we addressed this question using a suspension cell culture of *C. speciosa*, where cell morphology could be modulated between completely spherical (ratio of perimeter over long cell axis approaching π) and strongly elongate (ratio of perimeter over long cell axis approaching 2). As readout, the content of formononetin was quantified under these conditions (Wang et al. 2013; Zong et al. 2009). We find that formononetin is not detectable, irrespective of cell morphology or time after sub-cultivation (Table S1).

In contrast, MeJA a popular elicitor for induction of various secondary metabolites (Yukimune et al. 1996; Sharma et al. 2011), can induce the accumulation of formononetin in cells of *C. speciosa* (Fig. 6a). Interestingly, the hormonal activity of jasmonic acid was originally discovered because it can induce potato tube formation, a swollen organ where starch accumulates (Koda et al. 1991). However, in the cell culture of *C. speciosa*, there was no morphogenetic effect of MeJA whatsoever, although this trigger was efficient in activating formononetin. This indicates that MeJA acts as elicitor mimicking biotic or abiotic stress factors that have often been found to trigger the biosynthesis of secondary metabolites (Namdeo 2007; Sharma et al. 2011). Jasmonate signalling is integrated into numerous and quite diverse responses including responses herbivore injury, wounding, pathogens, but also developmental responses (for a comprehensive review see Wasternack and Hause 2013) as well as responses to abiotic stress, such as drought, or salinity (Riemann et al. 2015). This leads to the question, how to interpret the induction of formononetin accumulation in response to MeJA.

The biological function of secondary compounds accumulated in roots is often linked with the need to ward off attack by parasitic microorganisms dwelling in the soil. We therefore wondered, whether the biosynthesis of formononetin is linked with plant defence. Plant innate immunity is complex: A basal layer of defence is triggered by so-called pathogen-associated molecular patterns (PAMPs) that are shared by entire groups of microorganisms and that can be recognized by receptors in the plasma membrane. This basal, broadband layer of layer can also be accompanied by pathogen specific forms of immunity that are often linked with cell death. For necrotrophic pathogens, plants usually employ the basal, PAMP-triggered immunity, while for bio trophic pathogens that reprogramme the infected cell, a defence strategy culminating in cell death is more efficient. One of the most powerful PAMPs is flg22, a conserved peptide domain of bacterial flagellin. We observed that flg22 can induce formononetin, although to a much lower level as compared to MeJA (Fig. 6a). In contrast, when we tested NLP_{fo}, a member of Nep1-like proteins, virulence factors which can activate plant defence responses accompanied by cell death and tissue necrosis (Gijzen and Nürnberger 2006), we were not able to see any formononetin biosynthesis at all (Table S2). This result is consistent with findings in grapevine cells, where PAMP-triggered basal immunity (also triggered by flg22) and cell death-related immunity (induced by the bacterial elicitor harpin) were directly compared with respect to the role of jasmonate signalling (Chang et al. 2017): here, JA and its bioactive conjugate jasmonoyl-isoleucine (JA-Ile) accumulated rapidly during basal immunity, while there was no response in the context of cell death-related immunity. Since we also did not see any browning of cells, even after 20 h of treatment with MeJA, an indirect activation of formononetin production through activation of cell death is unlikely. Our results are therefore consistent with a model, where formononetin accumulates in response to basal defence conveyed by activation of jasmonate signalling.

Plant cell fermentation represents a promising and sustainable strategy for the biotechnological production of medically active plant compounds. The success of this strategy

not only depends on the efficient up scaling of biomass for industrial production, but also on the ability to trigger the biosynthesis pathway of interest. To identify proper elicitation protocols, it is important to elucidate the biological context for the activation of the secondary metabolites. Using the rare, and slowly growing species *C. speciosa*, we have addressed this biological context and were able to derive a protocol, which allows us to achieve formononetin accumulation in cell culture. With more genes involved in the biosynthesis pathway of formononetin were revealed (Li et al. 2016), it will greatly facilitate monitoring and eliciting the biosynthesis of this secondary metabolite. Not only formononetin, another active ingredient isoliquiritigenin was also detected after elicited by MeJA (data not shown), which indicated these compounds may be a general stress related result which signalling via jasmonate pathway. In intact root tissue, it takes months to swell and the mechanism behind will be more complex. Whether the mechanic tension during root swelling or environmental stresses can trigger jasmonate signalling for these secondary metabolites biosynthesis is still intriguing. Future work will now be dedicated to get deeper control over this process, for instance by directly addressing specific steps of the signalling pathway in order to promote and standardize product accumulation.

Author contribution statement FQ and PN designed the experiments, XJ analysed and interpreted the cell growth data; FQ, HS and PN wrote the manuscript; HS and HC analysed the content of formononetin by HPLC. FQ maintained the cell culture and elicited the cells. LL made the root sections. All authors read and approved the final manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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