Elicitation of Flavonoids in *Kalanchoe pinnata* by *Agrobacterium rhizogenes*-Mediated Transformation and UV-B Radiation

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Amalia Fkiara, Gregorio Barba-Espín, Rime El-Houri, Renate Müller, Lars Porskjær Christensen, and Henrik Lütken

Abstract

Kalanchoe pinnata from the stonecrop family (Crassulaceae) is a medicinal plant with high content of bioactive compounds. The plant is known for its anti-allergic, antioxidant, anti-inflammatory, antimicrobial and antibacterial activities, which are mainly attributed to flavonoids. Low yields of bioactive compounds in medicinal plants have led to new strategies for enhancing their biosynthetic capacity in order to increase the content of specialized metabolites. Herein, the accumulation of specialized metabolites in plants can be triggered by elicitation methods. In the current study, different elicitation strategies were conducted towards the enhancement of bioactive compounds in *K. pinnata* leaves. We investigated the effect of natural transformation with the *Agrobacterium rhizogenes* strain A4 as means of biological elicitation on the total content of flavonoids in the leaves of *K. pinnata.* Furthermore, the effect of supplemental UV-B radiation, as physical elicitor, was assessed on the total flavonoid content of both wild-type (WT) and *rol*-transformed plants. The combined effect of the two mentioned elicitation methods was also examined. The data showed that presence of *rol* genes resulted in an increase of 24% in the total flavonoid content compared to WT plants. The supplemental UV-B radiation increased the total content of flavonoids with 95% and 89% in the WT and the *rol*+ plants, respectively. Collectively, a synergistic

A. Fkiara · G. Barba-Espín · R. Müller · H. Lütken (✉)
Section for Crop Sciences, Department of Plant and Environmental Sciences, Faculty of Science, University of Copenhagen, Taastrup, Denmark
e-mail: hlm@plen.ku.dk

R. El-Houri · L. P. Christensen
Section of Biotechnology, Department of Chemical Engineering, Biotechnology and Environmental Technology, University of Southern Denmark, Odense, Denmark

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effect was shown as the combination of the two factors dramatically increased (133%) the total flavonoid content in *K. pinnata* leaves.

Keywords
A. rhizogenes · Elicitation · Flavonoids · *rol*+ · UV-B radiation

22.1 Introduction

In recent years, medicinal plants have been receiving increasing attention from the pharmaceutical industries as the interest in alternative therapies in the worldwide population is growing (Newman and Crag 2007). With the insecurity that emerged from the danger and toxicity of using some synthetic drugs and antibiotics, there has been a general increase in the perception that naturally derived products are safer than synthetic (Husain 2010). *Kalanchoe pinnata* (also known as *Bryophyllum pinnatum*), from the stonecrop family (Crassulaceae), is a popular plant both as ornamental and in folk medicine in many regions of the world. In the past, *K. pinnata* was used for alleviation or prevention of respiratory, stomach and hepatic problems, against tumour, kidney and spleen disorders, diabetes and vaginal diseases (Rahmatullah et al. 2010). Nowadays, *K. pinnata* is used for the anti-tumourous (Supratman et al. 2001), antihypertensive (Bopda et al. 2014), anti-allergic (Cruz et al. 2012), antimicrobial, antioxidant (Tatsimo et al. 2012) and anti-inflammatory (Nayak et al. 2010; Chibli et al. 2014) properties of its leaf extracts. These medicinal properties are attributed to the major classes of bioactive compounds in the plant, i.e. flavonoids, alkaloids and terpenoids, which constitute the active components of numerous herbal drugs (Kabera et al. 2014).

Agrobacterium rhizogenes-mediated transformation is a promising strategy for increasing the content of bioactive compounds in plants. *Agrobacterium rhizogenes* is a soilborne pathogenic bacterium causing the hairy root disease by infecting and inserting specific genes into the plant host's genome from wounded sites (White et al. 1985). The root-inducing (Ri) phenotype is the result of transfer, integration and expression of bacterial transfer DNA (T-DNA) in the transformed plant. In agropine strains of *A. rhizogenes*, the T-DNA is split into left (T_L) and right (T_R) fragments. The T_L-DNA comprises 18 open reading frames (ORFs) of which four *root oncogenic loci* (*rol* genes), termed *rol*A, *rol*B, *rol*C and *rol*D, are the major determinants for the development of characteristic 'hairy roots' at the infection site and are sufficient for obtaining the *A. rhizogenes*-induced phenotypes (Casanova et al. 2005; Christey 2001; Christensen and Müller 2009). Based on the naturally occurring *A. rhizogenes rol* genes, transformation without the use of recombinant DNA can be termed 'natural transformation', and plants derived from this platform are considered as non-GMO in several countries in Europe (European Union 2001). The expression of the *rol* genes, alone or combined, often leads to profound metabolic alterations that included increased accumulation of bioactive compounds. Moreover, transformed hairy roots often grow faster and are considered to be genetically stable

(Zhou et al. 2007). It has been reported that *Vitis amurensis* plant cells transformed with the *rol*B gene had a 100-fold increased production of the stilbenoid resveratrol (Kiselev et al. 2007), and *rol*C transformation of root cultures of *Atropa belladonna* increased the production of the alkaloids hyoscyamine and scopolamine 12-fold (Bonhomme et al. 2000). Moreover, *Panax ginseng* root cultures transformed with *rol*C displayed a production of ginsenosides of more than 6% dry weight, which is close to the maximum biosynthesis capacity (Bulgakov 2008).

As plants use sunlight for photosynthesis, they are as a consequence exposed to the ultraviolet (UV) radiation that is present in sunlight. Although the shortwave band of the terrestrial solar spectrum (UV-B radiation) accounts for less than 0.5% of the total solar energy reaching the surface of the earth, its high energy causes damages to RNA, DNA, proteins and lipids (Heisler et al. 2003). Therefore, plants have developed mechanisms to protect themselves from the harmful effect of UV-B radiation. One of the protective mechanisms is the accumulation of phenolic pigments in epidermal layers of leaves and stems, acting as filters that absorb UV-B light (Matsuura et al. 2012; Nascimento et al. 2015). Of all classes of phenolic compounds, flavonoids are regarded as the most relevant for UV protection.

In the present study, the role of *A. rhizogenes*-mediated transformation and UV-B radiation as enhancers of flavonoid contents was explored. Total flavonoids were determined by HPLC-DAD in wild-type (WT) and *rol*+ plants, prior and after exposure to supplemental UV-B radiation to determine potential synergistic effects.

22.2 Plant Material and Propagation

WT and *rol*+ *K. pinnata* plants were provided by Knud Jepsen A/S and used as maternal plants for further propagation. The *rol*+ plants were generated by *A. rhizogenes* (strain ATCC43057 containing plasmid pRiA4)-mediated transformation following Christensen et al. (2008). The maternal plants were grown in greenhouse for 9 months, in 2 L pots in a substrate mixture composed of peat supplemented with clay and silica (Weibulls Horto AB, Hammenhög, Sweden), under a 16 h day/8 h night photoperiod (23 °C/20 °C).

For propagation, 10-cm nodal cuttings were excised from the mother plants, dipped in auxin powder (Floramon A 1%, Novo Trade ApS, Odense, Denmark), planted in pots with the same substrate mixture and placed into the greenhouse at the above conditions. Eight-week-old propagated plants of both *K. pinnata* WT and *rol*+ were used in the experiments.

22.3 Climate Chamber Light Settings and UV-B Elicitation

The experiment was set up in a climate chamber (VEPHQ 5/2000, Heraeus Vötsch GmbH, Balingen, Germany) (16 h photoperiod, 28 °C during the day and 20 °C during the night). LED lamps (FL300 SUNLIGHT fixture, Fiona Lighting, Senmatic A/S, Søndersø, Denmark) were used as source of white light, supplying a

photosynthetic active radiation (PAR) of 180 μmol s^{-1} m^{-2} at plant height. The chamber was divided into two compartments by non-reflective screens. The first compartment included exclusively the use of white light from 7 to 23 h. In the second compartment, supplemental UV-B radiation was applied. Plants were placed at a distance of 2 m above the UV-B lamps (broadband lamps, Philips TL 40W/12 RS SLV) and irradiated with a UV-B intensity of 3 W m^{-2} during 5 h per day (from 10 to 15) for a week. UV-B light intensity was measured with a RM-12 Ultraviolet Light Meter equipped with a UV-B sensor (Opsytec Dr. Gröbel GmbH, Ettlingen, Germany). The irrigation was conducted manually (150 mL every second day). Two independent experiment repetitions displaced in time were conducted.

22.4 Sample Preparation

The pool of leaves from three plants was considered a biological replicate. After sampling, each biological replicate was ground into a powder in a Mortar Grinder Type RM 100 (Retsch GmBh, Haan, Germany), which was constantly kept cold by adding liquid N_2. One gram of produced powder was extracted twice with 5 mL methanol for 24 h at 5 °C in the dark, mixed frequently and centrifuged at 1000 rpm for 10 min. The supernatant was evaporated to dryness and redissolved in 1 ml methanol and filtered by a Q-Max syringe filter (13 mm Ø, PTFE membrane pore size 0.22 μm, Frisenette APS, Knebel, Denmark) prior to HPLC-DAD analyses.

22.5 Determination of Total Flavonoid Content (TFC) by High-Performance Liquid Chromatography-Diode Array Detection (HPLC-DAD)

The HPLC separations were carried out on a Luna C18(2) column (150 × 4.6 mm, 5 μm, 100 Å, Phenomenex, Allerod, Denmark) at room temperature. The mobile phase consisted of solvent A (aqueous with 0.1% formic acid) and solvent B (acetonitrile with 0.1% formic acid), and the following gradient system was used: 10–99% B (40 min), 99% B (34 min), 99–1% B (1 min) and 10% B (5 min). Flow rate was 0.8 mL min^{-1} and the injection volume 10 μL. Quercetin was used as standard. The retention time and ultraviolet spectra were obtained for chromatogram peaks at 254 nm. A quercetin calibration curve ($R^2 = 0.9974$) was used to quantify the TFC in the samples, constructed by plotting the integrated peak area at 254 nm against the calibration curve.

22.6 Statistical Analysis

All analyses were performed with six biological replicates. Data were subjected to statistical analysis using Microsoft Excel statistical package (version 15.16). Two-tailed Student's *t*-test with 95% and 99.9% degrees of confidence ($p \leq 0.05$ and

$p \leq 0.001$, respectively) was performed to indicate significant differences. Data were expressed as mean ± standard error (SE).

22.7 Elicitation of Flavonoids in *Kalanchoe pinnata*

In this study, the accumulation of flavonoids in *K. pinnata* plants was evaluated upon elicitation. Eight-week-old propagated *K. pinnata* plants were utilized in the experiment (Fig. 22.1). The TFC was calculated in leaves in WT and *rol*+ plants after 7 days of incubation with or without supplemental UV-B radiation (3 J m^{-2} during 5 h per day) (Figs. 22.2 and 22.3). In the WT plants, TFC was 232 ± 12 μg g^{-1}. In comparison, the corresponding content in *rol*+ plants was 287 ± 29 μg g^{-1}, representing a significant increase of 24% compared to the WT plants (Fig. 22.3). These results are supported by studies in which plants derived from *A. rhizogenes* transformation have the ability to produce higher amounts of valuable secondary metabolites in comparison with the wild-type counterparts (Giri and Narasu 2000; Oksman-Caldentey and Hiltunen 1996; Sevon and Oksman-Caldentey 2002). Integration of *A. rhizogenes* T-DNA in the plant's genome and corresponding expression of *rol* genes often alter the plant morphology (Chandra 2011). However, the *rol*+ lines used in this study did not show differences in terms of fresh weight and morphology of leaves compared to control lines (data not shown).

UV-B radiation induces photobiological stress in plants, which, among other effects, may lead to increased production of secondary metabolites (Schreiner et al. 2014). In the present work, for both *K. pinnata* plants, WT and *rol*+, the TFC increased upon UV-B light treatment compared to untreated plants. UV-B-treated WT plants displayed values of 452 ± 33 μg g^{-1}, representing an increase of 95% compared with the values of untreated WT plants. Similarly, the TFC of UV-B-treated *rol*+ plants was 541 ± 21 μg g^{-1}, which was 89% higher than that of the untreated *rol*+ plants (Fig. 22.3). When treated plants were compared with untreated plants, significant differences were observed at *p* values of ≤0.001 for both plant types. These results are in

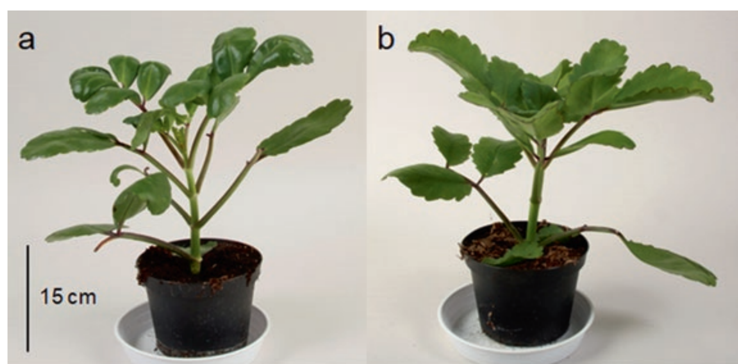


Fig. 22.1 Representative 8-week propagated WT (**a**) and *rol*+ (**b**) plants utilized in the UV-B elicitation experiment

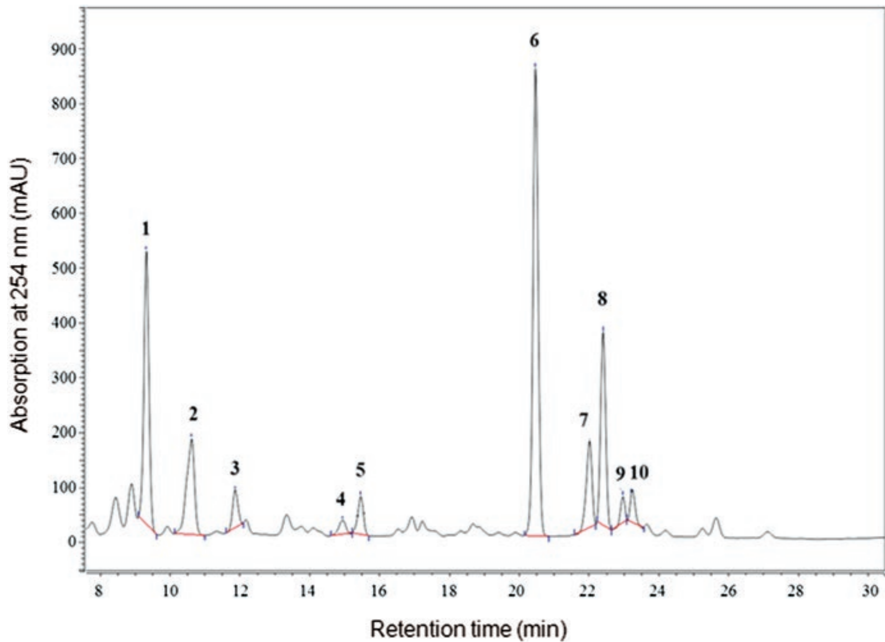
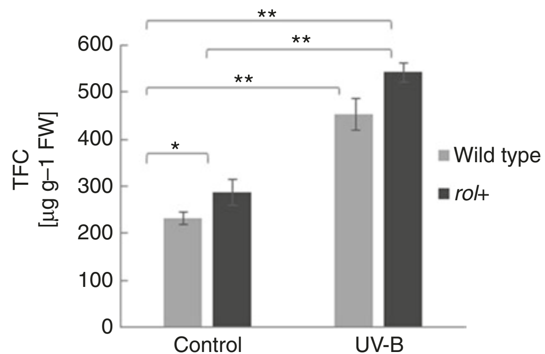


Fig. 22.2 Characteristic HPLC chromatogram of flavonoids in leaf extracts of *K. pinnata* recorded at 254 nm. Peak quantification was performed by plotting the integrated peak areas (1–10) against a quercetin calibration curve

Fig. 22.3 Total flavonoid content (TFC) in leaves of wild-type and *rol*+ *K. Pinnata* plants exposed to supplemental UV-B radiation (3 J m^{-2} for 5 h per day) or not (control) for 7 days. Data represent the mean ± SE, $n = 6$. (∗) and (∗∗) denote statistical significance according to two-tailed t-test at $p \leq 0.05$ and $p \leq 0.001$, respectively



agreement with previous studies that reported induction of flavonoids upon UV-B radiation. UV-B treatment increased total flavonol content in young petunia plants (Ryan et al. 1998) and the concentrations of two surface flavonols (calycopterin and 3′-methoxycalycopterin) in *Gnaphalium luteoalbum* plants (Cuadra et al. 1997). Mahdavian et al. (2008) reported that treatment with UV-B radiation increased proline, quercetin, rutin and anthocyanin concentrations in leaves of *Capsicum annuum*. Synthesis of phenolic substances such as anthocyanin and flavonoids was also

observed in UV-B-treated *Arabidopsis thaliana* seedlings (Bieza 2001). In another study in soybean (Middleton and Teramura 1993), it was indicated that UV-B light tolerance was positively correlated with the content of flavonoids. The effect of UV-B radiation was also investigated in vegetables and fruits during the vegetative and post-harvest period. Broccoli treated with supplementary UV-B radiation during the vegetative period increased the content of ascorbic acid, flavonoids and other phenolic compounds (Topcu et al. 2015). Ripe black currant fruits treated with short-term UV-B radiation after harvest showed an increase in the total content of phenolics and an altered phenolic composition (flavonols, anthocyanins, hydroxycinnamic and hydroxybenzoic acids) (Huyskens-Keil et al. 2007).

Additionally, the combination of *rol* transformation and UV-B light treatment resulted in the highest increase in the TFC (133%), when compared with untreated WT plants. To our knowledge, this represents the first reported synergy between the presence of *rol* genes and the use of UV radiation in the elicitation of secondary metabolites in in vivo plants. Hence, we propose that natural transformation followed by UV light elicitation can be a successful strategy for increasing the content of secondary metabolites in plants.

22.8 Conclusion

Agrobacterium rhizogenes-mediated transformation resulted in substantial increase of flavonoid content in *K. pinnata* leaves (24%). Furthermore, supplemental UV-B radiation for 7 days increased the content of total flavonoids in both WT and *rol*+ plants by 95% and 89%, respectively. Taken together, the synergistic effect of the two factors resulted in the highest increase of the total flavonoid content (133%).

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