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Original article

Early histological, hormonal, and molecular changes during pineapple (*Ananas comosus* (L.) Merrill) artificial flowering induction

Maita Eulalia Ávila Espinosa[a], Rafael Oliveira Moreira[b], André Almeida Lima[b], Solange Aparecida Ságio[c], Horllys Gomes Barreto[d], Sara Lazara Pérez Luiz[a], Carlos Eduardo Aragón Abreu[e], Ermis Yanes-Paz[e], Yanelis Capdesuñer Ruíz[e], Justo Lorenzo González-Olmedo[e], Antonio Chalfun-Júnior[b,*]

[a] Department of Basic and Specifics Sciences, University of Ciego de Ávila, Cuba
[b] Plant Molecular Physiology Laboratory, Biology Department, Federal University of Lavras (UFLA), Lavras, Minas Gerais, Brazil
[c] Federal University of Tocantins (UFT), Campus de Palmas, Palmas, Tocantins, Brazil
[d] Federal University of Tocantins (UFT), Campus Universitário de Gurupi, Gurupi, Tocantins, Brazil
[e] Bioplant Center, University of Ciego de Ávila, Cuba

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ABSTRACT

Natural flowering can cause serious scheduling problems in the pineapple (*Ananas comosus*) industry and increase harvest costs. Pineapple flowering is thought to be triggered by increased ethylene levels and artificial forcing of pineapple flowering is a common practice to promote flowering synchronisation. However, little is known about the early hormonal and molecular changes of pineapple flowering induction and development. Here, we aimed to analyse the molecular, hormonal, and histological changes during artificial pineapple flowering by Ethrel® 48 treatment. Histological analyses of the shoot apical meristem, leaf gibberellic acid (GA_3), and ethylene quantification were carried out during the first 72 h after Ethrel® 48 treatment. Expression profiles from ethylene biosynthesis (*AcACS2* and *AcACO1*), gibberellin metabolism (*AcGA2-ox1* and *AcDELLA1*), and flower development (*FT-like* gene (*AcFT*), *LFY*-like gene (*AcLFY*), and a *PISTILLATA-like* gene (*AcPI*)) genes were analysed during the first 24 h after Ethrel® 48 treatment. Differentiation processes of the shoot apical meristem into flower buds were already present in the first 72 h after Ethrel® 48 treatment. Ethrel® 48 lead to a reduction in GA_3 levels, probably triggered by elevated ethylene levels and the positive regulation *AcGA2-ox1*. *AcLFY* activation upon Ethrel® 48 may also have contributed to the reduction of GA_3 levels and, along with the up-regulation of *AcPI*, are probably associated with the flower induction activation. *AcFT* and *AcDELLA1* do not seem to be regulated by GA_3 and ethylene. Decreased GA_3 and increased ethylene levels suggest an accumulation of AcDELLA1, which may display an important role in pineapple flowering induction. Thus, this study shows that molecular, hormonal, and histological changes are present right after Ethrel® 48 treatment, providing new insights into how pineapple flowering occurs under natural conditions.

1. Introduction

Pineapple (*Ananas comosus* var. comosus) is one of the three most important tropical fruit in the world and is also an important ornamental and textile plant. One of the most undesirable characteristics of pineapple grown worldwide is the occurrence of natural flowering out of season, which can cause serious scheduling problems for pineapple growers. This can greatly affect the pineapple industry, since precocious flowering may lead to fruits that are too small to be marketable or too few in number, increasing harvest costs due to the relatively small percentage of these fruits (Bartholomew and Malrzieux, 1994; Van de Poel et al., 2009).

Pineapple reproductive development is induced by shortened day-length and cool night temperatures (Friend, 1981; Friend and Lydon, 1979; Gowing, 1961; Van Overbeek and Cruzado, 1948), and is assumed to be mediated by a burst in ethylene produc-

Abbreviations: ACC, 1-aminocyclopropane-1-carboxylic acid; ACS, ACC synthase; ACO, ACC oxidase; GA2-Ox, gibberellin-2-oxidase; GA_3, gibberellic acid; HAT, hours after treatment; RT-qPCR, quantitative RT-PCR.

* Corresponding author at: Plant Molecular Physiology Laboratory, Biology Department, Federal University of Lavras (UFLA), s/n - Cx. P. 3037, Minas Gerais, Brazil.
E-mail address: chalfunjunior@dbi.ufla.br (A. Chalfun-Júnior).

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tion, or increased ethylene sensitivity, in the shoot apical meristem triggered under these conditions (Bartholomew et al., 2003a). Artificial forcing of pineapple flowering is a well-established commercial practice that aims to synchronise pineapple flowering, being performed by using ethylene, ethylene releasing chemicals, such as ethephon [(2- chloroethyl) phosphonic acid], and acetylene-releasing calcium carbide (CaC_2) (Bartholomew et al., 2003b). Natural flowering inhibition may also be performed thorough the use of ethylene biosynthesis and action inhibitors, such as aviglycine (Kuan et al., 2005; Wang et al., 2007), and flowering induction may be achieved later at convenience by ethylene releasing agents. However, plant sensitivity to forcing depends on several factors such as cultivar, plant size, nutrients, and water stress. In addition, some of the agents used have had little success on flowering synchronisation (Kuan et al., 2005; Min and Bartholomew, 1996) and their misuse may increase production costs, instead of reducing them.

Although ethylene has been shown to promote flower induction in species from the *Bromeliaceae* family, such as pineapple, it can also inhibit flowering (Achard et al., 2007). Ethylene inhibits flowering by reducing bioactive gibberellins (GA) levels and enhancing DELLAs protein accumulation, which negatively regulates the floral meristem identity genes *LEAFY* (*LFY*) and *Suppressor of Overexpression of Constans 1* (*SOC1*) (Achard et al., 2007). On the other hand, prior to pineapple inflorescence emergence, the leaf basal-white tissue, known as basal achlorophyllous tissue, produces ethylene (Bartholomew et al., 2003a), and ethephon application can reduce the content of gibberellic acid (GA_3) in a long term (days) extent (Sheng-hui et al., 2013), contributing to pineapple flowering. These findings suggest that a reduction in GA levels and an increase in ethylene levels contribute to pineapple flowering, contrary to what is observed in Arabidopsis. However, it is not known how these hormones relate to each other at the early events of pineapple flower induction and upon ethylene application.

Along with plant hormones, temperature and photoperiod, other environmental and endogenous factors regulate the change from vegetative to reproductive growth in flowering plants, and five genetically defined pathways have been identified to control flowering in the model species Arabidopsis (*Arabidopsis thaliana)*: the vernalisation, photoperiod, gibberellin, autonomous, and endogenous pathways (Srikanth and Schmid, 2011). Signals from these pathways converge on floral integrators genes, such as *FLOWERING LOCUS T* (FT), the primary component of the florigen signal, which is thought to promote flowering together with the meristem-specific gene *FD* (Abe et al., 2005; Wigge et al., 2005), and *SOC1*, which in conjunction with *AGAMOUS-LIKE 24* (AGL24), promotes the expression of floral meristem identity genes, such as *LFY*, (Lee et al., 2008; Liu et al., 2008). LFY largely controls the subsequent switch to flower formation (Schultz and Haughn 1991; Wagner et al., 1999; Weigel et al., 1992), acting synergistically with the SQUAMOSA PROMOTER BINDING-LIKE (SPL) transcriptional factor SPL9 and DELLA proteins (Yamaguchi et al., 2014) to positively regulate the MADS box transcriptional factor APETALA 1 (AP1). *AP1* is expressed prior to the formation of the first flower, its expression is restricted to floral primordial that have committed to floral fate, it acts downstream and in parallel with *LFY*, and along with *LFY* jointly regulate early events during flower morphogenesis, including flower patterning (Bowman et al., 1993; Liu et al., 2009; Weigel and Meyerowitz, 1993,). *AP1* is a member from the A-class of the ABCE model, which postulates that the four regulatory functions (A, B, C and E) work combinatorically for proper organ formation of floral whorls, and along with *AP2*, confers sepal identity in the first whorl. Their activities overlap in the second whorl with the B-class proteins AP3 and PISTILLATA (PI), resulting in petal identity. Stamens are formed in the third whorl due to the combined activity of the B-class proteins together with the C-class protein AGAMOUS (AG), the latter specifying carpel development in the fourth whorl. The E-class proteins SEPALLATA1–4 (SEP1–4) play a crucial role as co-regulators in all four whorls (Pose et al., 2012).

Although some studies have shown that key genes in the control of flowering time in dicotyledonous species are conserved among monocot species (Peng et al., 2015; Ruelens et al., 2013;), only a limited number of them have been identified in monocot species, most of them in economically important ones, such as maize and rice (Lazakis et al., 2011; Zhang and Yuan, 2014; Yoshida and Nagato, 2011). Recent studies have identified several genes displaying high homology levels to flower development genes from Arabidopsis in pineapple (Liu and Fan, 2016; Lv et al., 2011; Lv et al.,2012a,b) and other species from the *Bromeliaceae* family (Li et al., 2015), suggesting that flowering control in these species may occur through similar mechanisms. Thus, in order to better understand the molecular, hormonal, and histological control of pineapple flowering during the first 72 h after artificial flowering induction, this study aimed access the histological changes of pineapple meristems apices, to determine the levels of GA_3 and ethylene from pineapple leaves, and analyse the expression pattern of genes related to ethylene and gibberellin metabolism, as well as, floral organ identity and development genes, in pineapple leaves in response to Ethrel® 48 application. The gene expression profile of seven different genes were studied, including two ethylene biosynthesis genes, one ACC synthase (*AcACS2*) and one ACC oxidase (*AcACO1*), a gibberellin inactivation gene (*AcGA2-ox1*), a putative pineapple DELLA gene (*AcDELLA1*) identified in this study, one FT-like gene (*AcFT*), one LFY-like gene (*AcLFY*), and a PISTILLATA −like gene (*AcPI*).

2. Material and methods

2.1. Plant material and culture conditions

The experiment was conducted under field conditions and was carried out using 12 months-old pineapple plants (*Ananas comosus* (L.) Merril cv. Smooth Cayenne), grown under field conditions in Cuba. Plants were not supplied with any nitrogen source during the previous 30 days from starting the experiment, and watering was suspended for two weeks before the flower induction treatment. Flower induction was performed in 300 plants (three replicates of 100 plants each) by spraying the shoot apex of the plants with a solution containing 350 mgL^{-1} Ethrel® 48 SL (2-chloroethyl phosphonic acid, Bayer Cropscience), 2% of urea, and $CaCO_3$ 0.5% (m/v) (based in previous studies). Treatment implementation was performed at 8 am, during non-inductive conditions of Cuba summer (June), and each plant was sprayed with 50 mL using a backpack system. The same number of plants was used for the control treatment, which consisted in the spraying the shoot apex of the plants with a solution containing 2% of urea and $CaCO_3$ 0.5% (m/v). Tissue sampling, which consisted of the basal achlorophyllous portion of D-leaves (defined as the youngest physiologically mature and fourth visible leaf from the shoot apex), was carried out before spraying the plants and at every 6 h from treatment imposition for the first 24 h (8a.m.; 2p.m.; 8p.m.; 2a.m.; 8a.m.) (molecular analyses), and at every 12 h for the first 72 h (8a.m.; 8p.m.) from treatment imposition (hormone analyses). At each sampling time, basal portions of D-leaves from three different plants of each treatment were harvested, with two technical replicates from each of the biological replicates. In addition to leaves, meristem apices were also sampled for inflorescence developmental stage determination. Fresh leaf tissues were used for ethylene quantification, while for GA_3 quantification and the gene expression analyses samples were immediately frozen in liquid nitrogen and later stored at −80 °C.