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Short communication

Ethylene and pollination decrease transcript abundance of an ethylene receptor gene in *Dendrobium* petals

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ABSTRACT

We studied the expression of a gene encoding an ethylene receptor, called *Ethylene Response Sensor 1* (*Den-ERS1*), in the petals of *Dendrobium* orchid flowers. Transcripts accumulated during the young floral bud stage and declined by the time the flowers had been open for several days. Pollination or exposure to exogenous ethylene resulted in earlier flower senescence, an increase in ethylene production and a lower *Den-ERS1* transcript abundance. Treatment with 1-methylcyclopropene (1-MCP), an inhibitor of the ethylene receptor, decreased ethylene production and resulted in high transcript abundance. The literature indicates two kinds of ethylene receptor genes with regard to the effects of ethylene. One group shows ethylene-induced down-regulated transcription, while the other has ethylene-induced up-regulation. The present gene is an example of the first group. The 5′ flanking region showed binding sites for Myb and myb-like, homeodomain, MADS domain, NAC, TCP, bHLH and EIN3-like transcription factors. The binding site for the EIN3-like factor might explain the ethylene effect on transcription. A few other transcription factors (RAV1 and NAC) seem also related to ethylene effects.

Introduction

Ethylene regulates fruit ripening and flower senescence. *Dendrobium* is among the flowers in which petal senescence is advanced by exogenous ethylene (Lerslerwong et al., 2009), which suggests that it is regulated by endogenous ethylene. In many flowers, including orchids such as *Dendrobium*, pollination induces early petal senescence by inducing an increase in flower ethylene production (van Doorn, 1997). In *Cymbidium* orchids, emasculation (the removal of pollinia) also increased ethylene production and hastened petal senescence (Woltering, 1990).

Ethylene binds to membrane-associated receptors. Five receptors (ETR1, ETR2, ERS1, ERS2 and EIN4) have been identified in *Arabidopsis*. These receptor proteins are negative regulators of the ethylene signal transduction pathway, i.e. they inhibit the pathway when not bound to ethylene (Shakeel et al., 2013). Treatment with ethylene reduced the amount of receptor protein. This might occur, according to one mechanism, through a decrease in mRNA abundance. This was found in *Os-ERS2* in rice (Yau et al., 2004) and *Ad-ETR1* in kiwi (Yin et al., 2008). By contrast, in other species ethylene did not affect the mRNA abundance of genes encoding ethylene receptors (e.g. *Arabidopsis ETR1* and *EIN4*, Hua et al., 1998; *Petunia ETR1* and *ETR2*, Dervinis et al., 2000) or induced an increase in transcript abundance (*Arabidopsis ERS1*, *ERS2*, and *ETR2*, Hua et al., 1998). These results seemed paradoxical if it is supposed that mRNA levels are positively correlated with protein levels. The paradox was solved by Kevany et al. (2007) who showed in tomato that ethylene induced an increase in mRNA abundance of *Le-ETR4* and *Le-ETR6*, but resulted in a decrease in the receptor protein levels, apparently by stimulating protein degradation in the 26S proteasome.

Here, we report on the expression of an *ERS* gene, isolated by Suwanagul et al. (2007), in petals of *Dendrobium* flowers. The aim of our study was to determine if the transcript abundance increases or decreases during petal senescence, in relation to effects of ethylene. Treatments were pollination, emasculation (removal of the anthers), exogenous ethylene, and 1-methylcyclopropene (1-MCP),

Abbreviations: 1-MCP, 1-methylcyclopropene; ERS, ethylene response sensor; ETR, ethylene triple response; EIN, ethylene insensitive.

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an inhibitor of ethylene perception. In order to find potential networks that regulate gene expression we also sequenced part of the 5′ flanking region of the gene.

Materials and methods

Plant material

Inflorescences of *Dendrobium* cv. Khao Sanan were obtained from a grower in Nakhon Pathom province, Thailand. Orchid flowers contain six tepals, of which one is called lip. Tepals will here be called petals. We distinguished floral bud stages B1–B3, describing the buds at the three tiers above the uppermost open flower on the inflorescence, at the time of harvest, whereby B1 is the smallest, more apical one. The stages OF1–OF4 describe the open(ing) flowers at four tiers, as indicated by the number, below the lowermost closed floral bud, at the time of harvest. OF1 is the most apical flower, which is just opening. OF2 flowers were half open, and OF3 and OF4 flowers were fully open. We also distinguished four stages of petal senescence during vase life: at S1 the vascular bundles change color (become darker); S2 indicates the onset of petal water soaking, at S3 the lip becomes yellow, combined with initial petal wilting, while S4 shows advanced petal wilting.

Upon arrival in the laboratory, some floral buds or open flowers were removed from inflorescences, leaving either about four buds or four open flowers. Fully open (OF4) flowers on inflorescences were hand-pollinated using pollinia of other open flowers of the same cultivar. Pollinia were removed by forceps (emasculation) from flowers at stage OF4, on different inflorescences. Ten inflorescences were used per treatment, using one flower per inflorescence. Inflorescences were placed in distilled water and were held at 25 °C, relative humidity (RH) of 70 ± 2% and a total photon flux density of about 15 μmol m^{-2} s^{-1}. Flower development was observed daily. The time to visible floral senescence was defined as the period from full opening to until clear petal wilting.

Treatment with ethylene and 1-MCP

Orchid inflorescences were placed in an airtight plastic chamber (37 cm × 47 cm × 35 cm). In ethylene treatment, ethylene gas was injected into the chamber to a final concentration of 0.4 μL L^{-1}. The inflorescences were exposed to ethylene for 24 h at 25 °C. 1-Methylcyclopropene (1-MCP) treatment was carried out using EthylBloc® powder (Smartfresh, Springhouse, PA, USA) in which 1-MCP was produced at a final concentration of 500 nL L^{-1}. The inflorescences were exposed for 3 h. Each experiment was carried out using three replications, consisting of three inflorescences per replication and one open flower per inflorescence.

Ethylene measurement

Detached flowers held in 10 mL vials containing 8 mL of distilled water were placed in airtight plastic chambers and kept at 25 °C for 2 h. Gas samples were taken from the headspace of each container and analyzed by gas chromatography (Shimadzu GC-8A, Kyoto, Japan) using an alumina separating column and a flame ionization detector. Three replications were used per treatment, each replication containing one flower.

Cloning of Den-ERS1 cDNA

Degenerate primers (Supplement Table 1) were designed from plant *ERS1* genes and used to amplify conserved regions of the trans-membrane domain II to GAF domain in reverse transcriptase polymerase chain reaction (RT-PCR). A cDNA sequence of *Dendrobium* Sonia 'Bom17' (Suwanagul et al., 2007) was used to design *Den-ERS1* specific primers (Supplement Table 1) to amplify the remaining open reading frame and 3′ end. Total RNA from petals was isolated by using TLES buffer (0.1 M Tris–HCl, 0.1 M LiCl, 0.01 M EDTA, 1% SDS) with 2% sodium sulfite and phenol/chloroform, precipitated using LiCl and re-precipitation with 2.5 volumes of 100% ethanol. All PCR products were cloned into pGEM®-T Easy (Promega). Sequencing of 14 clones was outsourced to a commercial company.

Cloning of the 5′ flanking region and the 5′ untranslated region

Genomic DNA was isolated from petals on open flowers, using TLES buffer containing 2% sodium sulfite and precipitated with equal volumes of 100% isopropanol. Thirty micrograms of genomic DNA was digested overnight at 37 °C with 30 U of *Xba*I (Fermentas). The digested genomic DNA was ligated overnight at 16 °C with annealed adaptors (Supplement Table 1; Cottage et al., 2001) using T4 DNA ligase (Fermentas). This ligation mix (10 μL) was used as template in a PCR reaction with an adapter primer and a nested adapter primer, in order to produce more template (Supplement Table 1). Amplified DNA fragments were cloned into pGEM®-T Easy (Promega). We then mixed the PCR product with competent cells for heat shock transformation. Sequencing of 12 clones was outsourced to a commercial company.

Sequence analysis and in silico analysis

Den-ERS1 nucleotide sequences in exon and intron regions were compared with those of rice *ERS1* (AY043031) and *Arabidopsis ERS1* (U21952) using Megalign (Clustal W method; Lasergene® version 7.2). The amino acid sequence was obtained by virtual translation. The sequence was analyzed for secondary protein structure using Protean (Lasergene® version 7.2). Alignments with ERS1 amino acid sequences from other species (the orchids *Phalaenopsis* sp. and *Oncidium* sp., and rice) were performed using CLUSTAL W (Larkin et al., 2007). The presence of *cis*-acting elements within the non-coding regions (5′ flanking region and 5′ untranslated region [UTR]) of *Den-ERS1* was determined using the MatInspector database (Matrix Family Library, version 9.1, September 2013, Genomatix Software, Munich Germany; http://www.genomatix.de) as described by Catharius et al. (2005).

RNA gel blot analysis

Twenty-five micrograms of total RNA was obtained as described above. RNA was denatured, and electrophoresed under denaturing conditions on 1% (w/v) agarose containing 5% formaldehyde for 2.5 h and the material was transferred onto a positively charged nylon membrane (Roche). The membrane was pre-hybridized, hybridized at 50 °C with *Den-ERS1* digoxigenin (DIG)-labeled probe for 16 h, and washed as recommended by the manufacturer (Roche). The signal was observed by chemiluminescence, using CDP-star (Roche).

Results

Sequence analysis of Den-ERS1

The cDNA sequence of the gene isolated from petals of *Dendrobium* cv. Khao Sanan flowers (accession number FJ628419) was identical with a gene previously identified in *Dendrobium* cv. Sonia Bom (Suwanagul et al., 2007). The deduced protein lacked a signal receiver domain, which assigns the gene to the ERS group. The gene was therefore called *Ethylene Response Sensor 1* (*Den-ERS1*).

The structure of *Den-ERS1* was compared with similar genes in *Oryza sativa* (*Os-ERS*) and *Arabidopsis* (*At-ERS*) (Supplement Fig. 1).