ABSTRACTS OF THE JOINT MEETING OF THE SPANISH AND PORTUGUESE MICROSCOPY SOCIETIES

XXIV CONGRESS OF THE SPANISH MICROSCOPY SOCIETY

XLIV ANNUAL MEETING OF THE PORTUGUESE SOCIETY FOR MICROSCOPY

SEGOVIA, 16-19TH JULY 2009

IE University
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LOCALIZATION OF ROS (REACTIVE OXYGEN SPECIES) AND NITRIC OXIDE IN OLIVE REPRODUCTIVE TISSUES BY STEREOMICROSCOPY AND CLSM.

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The olive tree is an important crop in the Mediterranean area. A consensus is beginning to be built on the presence of self-incompatibility mechanisms in most olive cultivars. However the molecular bases for this recognizing phenomenon are yet to be determined. Different reactive oxygen species (ROS) and nitric oxide (NO) are emerging as molecules able to module and control the complex signaling cascades regulating the interactions pollen-pistil in Angiosperms.

Different approaches have been used to determine the precise localization of some of these molecules in the olive pistil:

Either complete flowers or isolated pistils at different developmental stages were immersed in a solution containing 0.1 mg ml^{-1} of the indicator dye TMB (3,5,3´,5´-tetramethylbenzidine-HCl) in Tris-acetate, pH 5.0 [1]. The presence of H_2O_2 in these organs produced a blue-colored precipitate, which was detected using a Zeiss STEM1stereomicroscope, and was recorded with a Nikon Coolpix 4500 digital camera at a resolution of 2272 x 1704 dpi. The dynamics of the reaction was determined after quantization of the blue color produced in anthers and stigmas at different times of incubation. Color density was measured with using the Nikon EZ-C1 viewer (3.30) software, considering the basal color of the same plant structures before adding the TMB treatment.

The presence of both ROS and NO were detected using the fluorescent indicator dye DCFH2-DA (2´,7´-dichlorodihydrofluorescein diacetate) by using confocal laser scanning microscopy. Dissected floral buds or complete flower were immersed in 1ml of 50 µM DCFH2-DA in MES-KCl buffer (5 µM KCl, 10 mM MES, 50 µM CaCl2, pH 6.15) for 10 minutes, followed by a wash step in fresh buffer for 15 minutes. Parallel sets of samples were treated with a) sodium pyruvate 1M (H_2O_2 scavenger) in MES-KCl buffer for 30 min, followed by a wash step, or b) with 500 µM SNP (sodium nitroprusside, NO donor) in MES-KCl buffer and finally incubated with DCFH2-DA as above. Negative controls were treated only with MES-KCl buffer [2].

The NO indicator dye DAF-2 DA (diaminofluorescein diacetate) was applied to measure NO in flowers. Dissected buds or complete flowers were immersed in MES/KCl buffer for 10 min, transferred to 10 µM DAF-2 DA for 10 min, and finally washed with MES/KCl buffer for 15 min. Negative controls were treated the same, although with the buffer only [2].

Observations were carried out in a Nikon C1 confocal microscope using an Ar-488 laser source and different levels of magnification (20x to 60x). Small pinhole sizes (30 µm) were used even in combination with low-magnification, dry-objectives. Multiple optical sections were captured and processed to generate 3-D reconstructions of the whole stigma surface. 3-D reconstructions of small areas of the stigma surface were also generated from high-magnification immersion-objectives. The fluorescent

signal was obtained exclusively in the range of the 515-560 nm emission wavelengths with both fluorochromes, and was recorded in green color. Autofluorescence, mainly due to the presence of chlorophyll and other pigments was isolated and displayed in red.

The intensity of the fluorescence was also quantized for each organ at the different stages studied by using the Nikon EZ-C1 viewer (3.30) software.

We discuss why and how the described microscopical techniques are invaluable tools to determine the presence of these chemical species throughout the development of the anther and the pistil in the olive, a key question aimed at elucidating their important physiological roles.

This work was supported by research projects P06-AGR-01719 (Andalusian Regional Government) and BFU2008-00629 (Spanish MCyT). A Zafra thanks the CSIC for providing JAE grant funding.

References

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Fig 1. Complete flower of olive tree treated with TMB. The presence of H_2O_2 in the stigma and the anthers is showed by blue coloration (arrows).

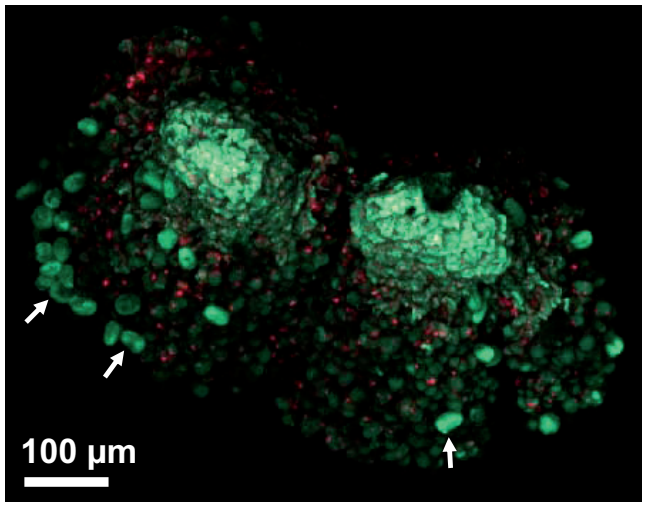

Fig. 2: Three-dimensional reconstruction of the apical end of the olive stigma. CLSM detection of NO by using DAF (green fluorescence). Pollen grains on the surface of the stigma are also positively labeled (white arrows).