Fluorochromes for Detection of Callose in Meiocytes of Olive (*Olea europaea* L.)

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ABSTRACT. The callosic wall which covers microsporocyte mother cells during meiotic division has been studied using different fluorochromes as alternatives to the widely used aniline blue. We have confirmed that both acridine orange and 4', 6' diamidino-2-phenylindole (DAPI) produce a fluorescent response to callose which is comparable in specificity and intensity to that of aniline blue; therefore, they can be used to study callose wall formation. Staining properties of these fluorochromes, as well of those of curcumin and sirofluor, reported earlier as fluorescent stains for callose, are discussed. We also discuss the efficacy of the combined use of sirofluor and DAPI to study particular aspects of the deposition of callose.

Key words: acridine orange, callose, DAPI, fluorochromes, microsporocytes, olive, sirofluor

Callose is a polymer of (1→3)- β -linked glucose residues (Kessler 1958) which has been identified in different cellular locations in plants (Currier 1957). During microsporogenesis, each microsporocyte mother cell (MMC) secretes a special cover made of this material, which persists throughout meiosis until the tetrad stage. At this time, the callose wall begins a phase of degradation at the end of which microspores are released into the locule of the anther (Bhandari 1982, Paccini 1994). Although its biological significance is not well understood, the most widely accepted theories are that the callose wall protects and isolates

meiocytes, and acts as a template for primexine deposition (Heslop-Harrison and Mackenzie 1967, Barskaya and Balina 1971, Waterkeyn and Bienfait 1971).

Cytochemical methods for callose detection (IKI-aniline blue, resorcinol blue, lacmoid, etc.) have been reviewed by Eschrich (1956). Other methods have since been described, such as those of Ulrichova et al. (1976), and include the use of coraline, benzoazurine, azurine or Ponceau S. The use of aniline blue with fluorescence microscopy, introduced by Arens (1949) and popularized by Currier and Strügger (1956), is the most widely used method for detecting callose.

Nevertheless, new methods based on either fluorescent or nonfluorescent probes are now used for this purpose. Evans and Hoyne (1982) isolated a fluorochrome, sirofluor, from aniline blue commercial samples which shows a specific reaction with callose. Stockert et al. (1989) have used a natural dye (curcumin) to obtain a specific signal from the callose. Antibodies have also been used to locate (1→3)- β -glucans (Meikle et al. 1991, Hussey et al. 1992).

We present here results we obtained after using various fluorochromes for histochemical detection of callose in MMCs. Their specificity and the possibility of their combined use are reported also.

MATERIALS AND METHODS

Flowers of olive (*Olea europaea* L.) in different developmental stages were dissected in a Petri dish containing cold (4 °C) fixative (4% paraformaldehyde, 3% glutaraldehyde in 0.1

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M cacodylate buffer, pH 7.2). The anthers were fixed using this same fixative for 2 hr at 4 C. A slight vacuum was applied for 10 min during this period. The samples were then dehydrated through an ethanol series and embedded in Epon.

The experiments were performed using thin sections in (1 μ m) of the anthers on glass slides. Sections on the slides were pretreated using a freshly made concentrated solution (10 M) of sodium hydroxide in methanol for 2 hr to make the resin permeable. After pretreatment, the slides were stained in the dark using a solution of fluorochrome for 30 min to 2 hr, mounted in citifluor-glycerol-phosphate buffered saline (PBS) (Agar Scientific Ltd., Stantard, UK) and observed in a Zeiss Axioptan photomicroscope equipped for epifluorescence microscopy (HBO 50 W mercury lamp, BP436/8, FT460 LP470 filters for violet-blue excitation; BP450-490, FT510, LP520 filters for blue excitation and BP365/11, FT395, LP397 for ultraviolet excitation).

The fluorochromes were used in solutions as follows:

Aniline blue (EMscope Laboratories, Ashford, UK) 0.01% in 1.5% K_2PO_4H , pH 8.6. Staining time, 30 min. Blue excitation.

Curcumin (Sigma Chemicals, St. Louis, MO) 0.2 mg/ml in 5% ethanol. Staining time, 2 hr. Blue excitation.

DAPI (Sigma) 10 μ g/ml in distilled water. Staining time, 1 hr. Ultraviolet excitation.

Acridine orange (Sigma) 0.1% in PBS pH 6.2. Staining time, 1 hr 30 min. Violet-blue excitation.

Sirofluor (Biosupplies Australia, Parkville, Australia) 0.025 mg/ml in distilled water. Incubation time, 30 min. Blue excitation.

Sections of the same samples used for fluorochrome testing were stained with toluidine blue to determine the meiotic stage of the anthers.

The combined use of sirofluor and DAPI was tested by squashing previously fixed anthers (3:1 ethanol:acetic acid fixative) in a mix of both DAPI and sirofluor solutions in equal proportions. Once squashed, the anthers were covered with a coverslip and observed. Both fluorochrome solutions were as described above, but 1% Triton X-100 was added to the DAPI solution to increase the permeability of the cells. In this case, ultraviolet excitation was used.

RESULTS

After treating the sections with aniline blue, the special wall of callose around the MMCs shows a yellow fluorescence clearly defined in the different meiotic stages in which it appears (Figs. 1 and 2). Despite a certain degree of autofluorescence, the signal of this fluorochrome is rather high. This fluorochrome can be used to study the first deposits of callose which appear in late pachytene, since at this stage callose and autofluorescence can be distinguished easily (Fig. 2).

Treating slides with acridine orange solution produces a yellow/orange metachromatic fluorescence in the areas of callose deposition that makes this fluorochrome useful for studying callose formation. Although the fluorescence level in tissue components not containing β -glucans is relatively high (Figs. 3 and 4), this fluorochrome can be used to study the initial deposits of callose in pachytene-diplotene (Fig. 4). Nuclear DNA, nucleoli and cytoplasmic components also fluoresce slightly when higher

Figs. 1-10. Bar represents 10 μ m, except Figs. 3 and 4 where it represents 25 μ m.

Fig. 1. Callose deposits stained with aniline blue (arrows). Tetrads before microspore release.

Fig. 2. Early callose deposits at late pachytene (curved arrows) stained with aniline blue.

Fig. 3. Acridine orange stain. Callose deposits at the tetrad stage (arrows).

Fig. 4. Acridine orange stain. Early deposits at pachytene-diplotene (curved arrows). Note fluorescence in cell walls, nucleus and cytoplasm of the cells.

Fig. 5. Tetrads stained using DAPI. Callose fluoresces blue (arrows). The DNA is not appreciably stained using this method.

Fig. 6. Curcumin staining of tetrads. Weak greenish fluorescence is observed in the callose (arrows). A high level of background can be seen, particularly in the nucleus.

Fig. 7. Sirofluor staining. Callose deposits at metaphase I (arrows).

Fig. 8. Sirofluor staining. Very early deposits of callose at pachytene (curved arrows).

Figs. 9 and 10. Double staining using sirofluor and DAPI under ultraviolet excitation. Callose fluorescence is weaker than under blue excitation (arrows), but is still clearly distinguishable. The chromatin (Chr) fluoresces in blue, whereas the cytoplasm (Cy) shows slight background which allows the observation of an interphase between the callose wall and the protoplasm (asterisks).

Fig. 9. Cell at prometaphase I.

Fig. 10. Tetrads before microspore release.

Table 1. Characteristics of the Fluorochromes Used

Fluorochrome	Signal	Background	Rate of Fading
Aniline blue	*****	**	***
Curcumin	***	*****	***
Acridine orange	*****	***	***
DAPI	*****	****	**
Sirofluor	*****	*	**

Very high (*****), High (****), Moderately high (***), Medium (**), Moderately low (*), Low (*).

concentrations of the dye, extended pretreatment or staining (results not shown), and/or photographic exposure (Fig. 4) are used.

DAPI staining has similar characteristics (Fig. 5), that is, a high intensity signal for callose and a high level of fluorescence in the cell walls, greater in this case than that of acridine orange. Because of this, DAPI does not allow good discrimination of the callose in the first stages of its deposition. Similar to acridine orange staining, extended DAPI staining of sections produces fluorescence in the nuclear material (results not shown).

The use of curcumin as the fluorochrome also produces secondary yellowish fluorescence in the callose (Fig. 6). Using the described procedure, a high level of background appears in the protoplasm and cell walls of MMCs. The intensity of the signal in the callose is not as high as with aniline blue, and use of curcumin in early meiotic stages is not recommended.

The fluorochrome sirofluor combines a strong signal with low background fluorescence (Figs. 7 and 8). As a result, it heightens the contrast, allowing distinction between the callose and the autofluorescence of the cell walls. This fluorochrome can be used to detect what may be considered the first deposits of callose around MMCs (Fig. 8).

If both DAPI and sirofluor are used under ultraviolet excitation, the callose appears yellow-green with weaker fluorescence than under blue excitation. The chromatin of the meiocytes is blue, and a slight autofluorescence appears in the cytoplasm. In this way, the boundary between the protoplasm of meiocytes and their covering of callose can be distinguished readily (Figs. 9 and 10).

DAPI and sirofluor are relatively resistant to fading (Table 1). Acridine orange and aniline blue are moderately likely to lose intensity, while curcumin fades extremely rapidly. When using curcumin, it is very difficult to obtain photomicrographs, and high sensitivity films should be used.

DISCUSSION

The use of fluorochromes for labeling cellular components presents several advantages (Kasten 1981) including increased sensitivity, the possibility of using concentrations of stain which are nontoxic and therefore suitable for *in vivo* studies, and the possibility of their combined use with transmitted light microscopy. On the other hand, multiple labeling is becoming a powerful tool for detecting different antigens or cellular components simultaneously. Most of these studies involve the use of different fluorochromes and/or labeled antibodies in conjunction with appropriate narrow-band filter systems, confocal microscopy or even normal optical microscopy. Therefore, the acquisition of information concerning the interaction of well-known fluorochromes with cellular components and data concerning the use of new fluorochromes is of great importance.

The specificity of the dyes used in this work varies greatly. Aniline blue and sirofluor have been widely analyzed in studies of complexing in solution and, in the case of sirofluor, in sections of material prepared for fluorescence microscopy (Stone et al. 1984). Basically, both induce strong fluorescence with linear (1→3)- β -D-glucans, showing minor fluorescence with some other glucans (Evans and Hoyne 1982, 1984, Evans et al. 1984, Wood and Fulcher 1983). Curcumin reacts with callose as well as with different cellular components of both animal and plant origin including mucin granules, cartilage matrix, elastin and cell walls (Stockert et al. 1989).

To our knowledge, neither DAPI nor acridine orange has been used previously for the histochemical detection of callose. DAPI is considered a very specific quantitative stain for DNA (Coleman and Goff 1985) whereas acridine orange has been widely used to stain and differentiate nucleic acids (Armstrong 1956, Haldrup

and Bruun 1993). These similar properties allow the use of both dyes for purposes such as counting bacteria (Suzuki et al. 1993) or chromosome banding (Suja et al. 1994, Bella and Gosalvez 1994). Acridine orange, however, has been used for many other applications such as staining of fungi (Pickett et al. 1960) and plant viruses (Hooker and Summanwar 1964), and investigating pollen or bacterial viability (Georgieva and Kruleva 1993, Binnerup et al. 1993).

The broad spectra of staining by these dyes, particularly those of the curcumin and acridine orange, can be explained by the existence of several specific mechanisms of staining; however, the possibility of nonspecific or alternative interactions with callose has to be taken into account. In this context, interactions of acridine orange and other cationic dyes with bacterial polysaccharides have already been described. Some of these interactions include changes in the spectrophotometric and spectrofluorometric properties of the dyes, as well as metachromasia in a particular case (Mitra et al. 1993). The highly hydrophilic character of callose would, in our opinion, be one of the main causes of the staining, due to the fact that the aqueous dye solution would be "trapped" in the callose matrix. Further studies of complexing in solution are necessary to establish the nature of these interactions.

Fluorescent DNA probes such as ethidium bromide and Hoechst 33258 have already been used in conjunction with sirofluor to monitor nuclear events during pollen tube growth (Hough et al. 1985). As indicated by these authors, one of the main advantages of sirofluor over aniline blue is its binding specificity at neutral rather than alkaline pH, making it suitable for vital staining.

The interphase between the protoplasm of meiocytes and their cover of callose, which is visible using combined DAPI and sirofluor staining, has been described previously (Alché et al. 1990). These authors suggested the interphase layer is composed of callose precursors, perhaps with a low degree of polymerization, and therefore unable to show fluorescence after aniline blue staining. The double labeling technique described here, which shows this layer as a region lacking fluorescence, is therefore a valuable tool for studying its development throughout microsporogenesis.

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