Investigation of the Effects of the Yr-18 Durable Adult-Plant Resistance Gene in the Near Isogenic Line of Spring Wheat to Stripe Rust with Confocal Laser Scanning Microscopy

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ABSTRACT

Confocal laser scanning microscopy (CLSM) is a revolutionary advance in the field of light microscopy which, in recent years, has been used on interaction studies between host and pathogen. This study carried out on the flag leaves of the susceptible spring wheat cultivar (Triticum aestivum L.) known as Thatcher and its near isogenic line with the Yr-18 adult-plant resistance gene to stripe rust fungus (Puccinia striiformis f. sp. tritici), by confocal laser scanning microscopy. A suspension of fresh urediniospores of stripe rust, isolate SR99-UA (race, 70E 128) in a light mineral oil (Soltrol, 170) was sprayed on the flag leaves in vitro, then four segments were sampled at 2, 4, 8, 12 and 18 days after inoculation. These segments were further divided into 1-2 cm. For detailed observation, two fluorescent dyes Acridin orange and X-Rodamin-1, known to be nucleic acid and calcium binding probes, were used, respectively. CLSM made it possible to visualize host/pathogen interaction in serial without sectioning at a three dimensional level from adaxial to abaxial leaves. According to the results, no difference in pre-penetration behavior of the fungus in the two genotypes was observed. During the early stages of penetration, host cell necrosis was occasionally seen only on the resistant genotype. Few dead cells were observed on the susceptible host 12-18 days after inoculation. However, by this time, the resistant host had a markedly high number of dead cells; there were numerous necrotic areas or pustules resembling hypersensitive response. In the infected areas and around the pustules of the resistant host a remarkable red color was observed by the presence of a thick fluorescence rich calcium layer. The intensity of calcium fluorescence in the non-infected areas of both genotypes and in the infected areas of the susceptible host were nearly the same, indicating calcium mobilization as part of the defensive response in resistant near isogentic lines containing the gene Yr-18. This result indicated that calcium mobilization at the point of challenge by the pathogen played an important role in the near isogenic resistance line of Thatcher in the wheat stripe rust pathosystem. This is the first report on calcium mobilization in a cereal rust pathosystem as a part of the resistance response.

Keywords: Calcium mobilization, Confocal microscopy, Stripe rust, Wheat.

INTRODUCTION

Stripe (yellow) rust of wheat caused by Puccinia striiformis West. f. sp. tritici Eriks et Henn is one of the major diseases of wheat (Triticum aestivum L.) in various regions of the world (Roelfs et al., 1992). Confocal microscopy is a revolutionary advance in the field of light microscopy. This new type of microscopy offers biologists a unique form of cell and/or subcellular visualization. The principle of mi-

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croscopy is that it permits “optical sectioning” rather than mechanical sectioning of both thin and thick microscope samples in fluorescence and reflection with a conventional light microscope. This microscope can be successfully applied to variety of pathogen/host interaction (Czymmek et al., 1994). The first confocal image was generated in 1884 by Nipkow (Inoue, 1989). In 1957 Minsky a post-doctoral Fellow at Harvard University, developed a stage-scanning confocal microscope of our present instruments (Minsky, 1988). Until the mid-1980s, developments in computing, electronic and laser technology were not adequate to support reliable commercial services (Shotton, 1989).

In recent years, CLSM has been used more in experimental research programs. For example, Laurincik et al. (2000) studied nucleolar proteins in pre-implantation bovine embryos produced in vitro. Xu and Heath (1998) also studied calcium in signal transduction during the hypersensitive response caused by basidiospore-derived infection of the cowpea rust fungus using confocal laser scanning microscopy.

During their lifetime, plants are subjected to thousands of microbial attacks. Active plant defenses against these microbes include the formation of mechanical barriers and increases in the levels of potentially antimicrobial molecules (Lamb et al., 1989; Dixon et al., 1994) and are usually accompanied by the rapid death of one or more plant cells. This rapid, localized cell death associated with disease resistance is known as the hypersensitive response (HR) (Xu and Heath, 1998). Increasing evidence indicates that the HR is a form of programmed cell death (Greenberg et al., 1994; Heath, 1998).

Recognition of a pathogen by the plant presumably leads to signal transduction cascade in plant cells (Ebel and Casio, 1994), but signal transduction pathways in plants have yet to be clearly elucidated. In plants, many stimuli are mediated by elevation of cytosolic free calcium ([Ca^{2+}]_{c}) (Bush, 1995). The involvement of calcium in responses of cell cultures or protoplasts to microbial compounds (elicitors) has been demonstrated (Mahady and Beecher, 1994; Messian and Van Custen, 1994; Suzuki et al., 1995; Tavemier et al., 1995; Ishihara et al., 1996; Levine et al., 1996). Experimental results indicated that elicitors may affect the activity of plasma membrane calcium channels (Gelli et al., 1997; Zimmermann et al., 1997).

Xu and Heath (1998) and Heath et al. (1997) examined cytosolic calcium levels during HR of epidermal cells in cowpea plants with an obligate pathogen and causal agent of cowpea rust disease, by using CLSM in conjunction with a high fluorescence calcium dye. Cells invaded by race 1 of cowpea rust fungus showed HR in resistant hosts but did not cause any detectable resistance response, or cell death, in the susceptible genotype. They showed that elevation of [ca^{2+}]_{c} was detectable in the epidermal cells of resistant plants before completed penetration of the plant epidermal wall and before other detectable cytoplasmic manifestation of the HR. The aim of this investigation was to elucidate the role of cytosolic free calcium during the hypersensitive response of a near isogenic line of spring wheat resistant to the yellow rust fungus.

**MATERIALS AND METHODS**

This study was carried out on the flag leaves of a susceptible spring wheat genotype (*Triticum aestivum* L.) known as Thatcher and its *Yr*-18 gene containing a near isogenic line with adult-plant resistance to stripe rust (yellow rust) fungus (*Puccinia striiformis*) using confocal laser scanning microscopy (Molecular Dynamics Multiprob Laser-Argon/ Crypton; Saftwar-Image space 3.2). One of the Canadian stripe rust isolates, namely SR99-UA, multiplied on seedlings of the susceptible cv. Avocet in this study. For doing the experiment at the adult-plant stage, 10 seeds from each genotype were sown in 12.5 diameter pots containing compost (Metro-Mix 292, Ltd,
Terra), and inoculated when the flag leaf fully expanded, about 8-9 weeks after sowing. A suspension of fresh urediniospores in a light mineral oil (Soltrol 170) at a concentration of 5 mg/ml was sprayed on the leaves. A light coating of oil was enough to ensure good infection. Inoculated plants were left at least one hour for the oil evaporate off the leaves. Then, the plants received a light spray of water, were covered in plastic bags to maintain high humidity and placed in dark at 10°C for one day. One day after inoculation, the plants were moved to a growth chamber set at 15°C with a 16h photoperiod and a relative humidity within the range 60-70%. The light intensity was approximately 8000 Lux at seedling height.

Samples for observation were taken two days after inoculation and then every subsequent two days up to 18 days during active sporulation. At each sampling, 4 cm long leaf segments were taken 5-9 cm from the leaf tip.

For observation by CLSM, two fluorescence dyes, namely Acridine orange (Molecular Probes A 1301) which is a nucleic acid binding and X-Rodamine-1 (Molecular Probes X-14210) which is a calcium binding fluorescence probe, were used in this study.

Inoculated leaf segments were excised and cleared in ethanol (Et.): methyl salicylate (MS) series; 1 Et.: 3 MS; 1 Et.: 1 MS; 3 Et.: 1 MS; 30% Et.; 70 % Et. and 95 Et. for 30 minutes in each concentration. Finally, the segments were stored in absolute methyl salicylate in teflon cupped glass vials.

The cleared leaf samples were then stained in 0.02 % Acridine orange in sodium acetate. Acridine orange interacts with DNA and RNA by intercalation or electrostatic attraction. X-Rodamin-1 in 10^{-18} micromolar in dimethyl sulfoxide (DMSO) is also used as a long-wavelength calcium indicator in cell and tissue. On the day of observation the cleared leaf samples were put in 10^{-18} micromolar X-Rodamin-1 dye for 30 minutes, washed in DMSO for 30 minutes and then mounted in DMSO on microscope slide for CLSM observation.

**RESULTS AND DISCUSSION**

Confocal laser scanning microscopy is versatile and easy to use, because fluorescence imaging is a very powerful tool. The results of this study and that of other researchers have shown that confocal imaging is valuable in fungal studies on a variety of subjects from spore development to intracellular signaling citation. Investigation with CLSM made it possible to visualize cellular and sub-cellular events related to pathogen/host interaction at three dimensional levels.

The percentage of spore germination and pre-penetration development of the pathogen in both susceptible and resistant genotypes was nearly the same. In a susceptible host, the plant nucleus becomes closely associated with the pathogen and, at the later stage, eventually moves into neighboring epidermal and mesophyll cells. This process does not cause any death of the cells. This observation was similar to the results of Heath (1989). In the resistant host, haustoria formation and growth of hyphae through the mesophyll cell lead to a collapse of the tissue in the host. These results are similar to those finding of Ayesu-Offeri and Clare (1970) and Lyngs-Jorgensen et al. (1993) on barley infected with scald (Rhynchosporium secalis), and also similar to the observations of Xu and Heath (1998) on cowpea infected with rust fungus (Uromyces vignae).

On the surface of the necrotic areas/pustules of the resistant genotype these were marked by the presence of a thick fluorescence rich calcium in red color (Figures 1 and 3) indicating a strong mobilization of calcium in response to infection. Various stages in hypersensitive cells death were recognized cytologically as a resistance response in this study. These observations are similar to those of Chen and Heath (1991) and Heath et al. (1997). As mentioned earlier, elevation of the calcium level was a host-resistance defense process and was not affected by the pathogen.

On the surface of the infected areas/pustules of the susceptible host, a low intensity
of calcium fluorescence with a high amount of spores were observed (Figures 2 and 4). The intensity of calcium fluorescence in the non-infected areas of both genotypes, and in the infected areas of susceptible genotype were nearly the same (Figure 5) supporting the process of calcium mobilization as a part of the defense response in the resistant genotype containing the gene Yr-18. These results indicated that calcium mobilization at the point of challenge by the pathogen plays a role in host resistance in the wheat stripe rust pathosystem.

These results are similar to those observed by Xu, and Heath, (1998) on the role of calcium in signal transduction during the hypersensitive response caused by basidiospore-driven infection of the cowpea rust.
fungus. Their calcium levels ratio analysis and imaging studies demonstrated a consistent increase of $[\text{Ca}^{2+}]$ in the resistant near isogenic line but not in the susceptible spring wheat cells. There are several reports on the role of calcium in the signal transduction leading to a plant defense response such as those of Mahady and Beecher, 1994; Messiaen and Van Custen, 1994; Suzuki et al., 1995; Tavenier et al., 1995; Ishihara et al., 1996; and Levien et al., 1996 in which cell cultures (cytocolic) were treated with elicitors. These results suggested that the increase in the $[\text{Ca}^{2+}]$ level related to the timing of the pathogen invasion and not to the timing of the HR occurrence. Furthermore, the results also indicated that calcium mobilization exerts a major role in the mode of action of the Yr-18 gene in mediating stripe rust resistance in spring wheat. This is the first report on the mobilization of calcium in a cereal-rust pathosystem as a part of a resistance response.

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بررسی اثرات زن 18-26، عامل ایجاد مقاومت پایدار در بروزوزنیکی لاين گندم بهره‌برداری از ژن Yr-18 در زنگ توده‌ای با استفاده از میکروسکوپ اسکن لیزرویی CLSM

چکیده

پژوهش‌های اخیر نشان داد که در این سال‌های اخیر، استفاده از ژن‌های زنگ توده‌ای در کاهش دردناکتی در گندم بهره‌برداری از این ژن‌ها مطرح شده‌است. بررسی‌های اخیر نشان داده‌اند که در حالت‌های مخصوصی از واقعه‌های مصرف و تولید نیترات به وجود آمده در حالت‌های مخصوصی، این ژن‌ها به صورت فعالیت‌دار می‌باشند. نتیجه‌گیری‌های اخیر نشان داد که در حالت‌های ژن‌های زنگ توده‌ای، مقاومت حساسیت به فیتوکاتیون‌ها می‌باشد. نتایج حاصل از لایه‌های این ژن‌ها نشان داد که در حالت‌های مخصوصی از واقعه‌های مصرف و تولید نیترات به وجود آمده در حالت‌های مخصوصی، این ژن‌ها به صورت فعالیت‌دار می‌باشند. نتیجه‌گیری‌های اخیر نشان داد که در حالت‌های ژن‌های زنگ توده‌ای، مقاومت حساسیت به فیتوکاتیون‌ها می‌باشد.

سخنرانی‌های این ژن‌ها نشان داد که در حالت‌های مخصوصی از واقعه‌های مصرف و تولید نیترات به وجود آمده در حالت‌های مخصوصی، این ژن‌ها به صورت فعالیت‌دار می‌باشند. نتیجه‌گیری‌های اخیر نشان داد که در حالت‌های ژن‌های زنگ توده‌ای، مقاومت حساسیت به فیتوکاتیون‌ها می‌باشد.

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