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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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ASTRACT

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 aboxial 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 isogenic 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 ($[Ca^{2+}]f$ (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 cytocolic 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 $[ca^{2+}]i$ 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 stiiformis*) 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,

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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 5-9cm 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

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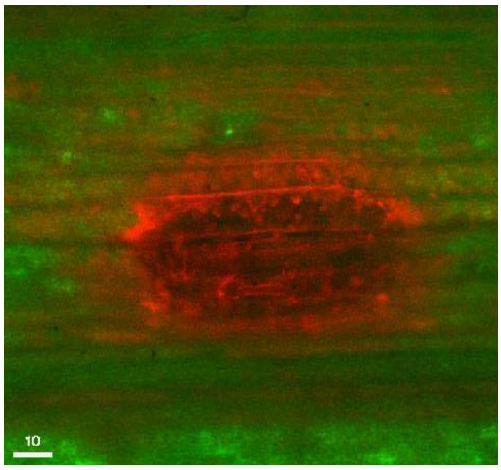

Figure 1. Intensity of high calcium fluorescence in/ around the necrotic pustules areas of the resistant host infected with P. *striiformis*, 18 days after inoculation.

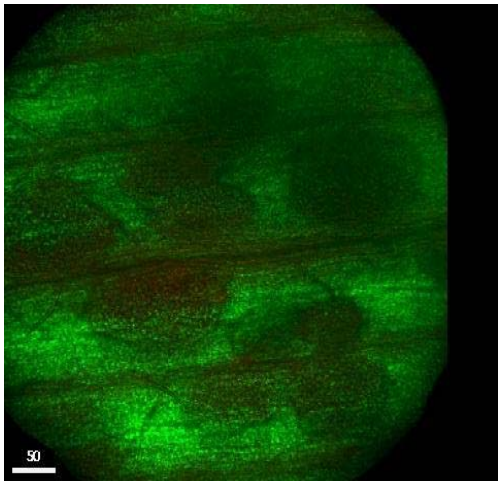

Figure 2. Low calcium fluorescence intensity in/around the pustules of the susceptible host infected with P. *striiformis*, 18 days after inoculation.

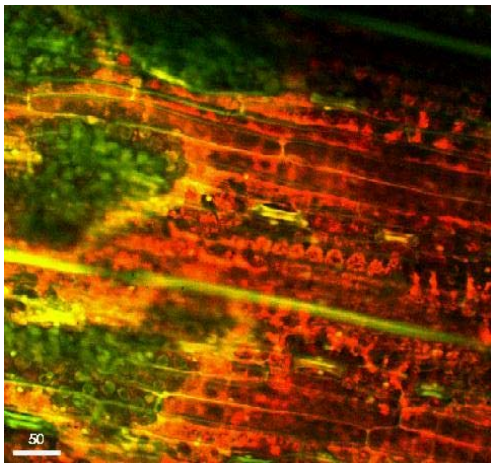

Figure 3. Thick fluorescence of rich calcium in red color, marked on the surface of the necrotic areas/pustules of resistant genotype infected with P. *striiformis*, 18 days after inoculation.

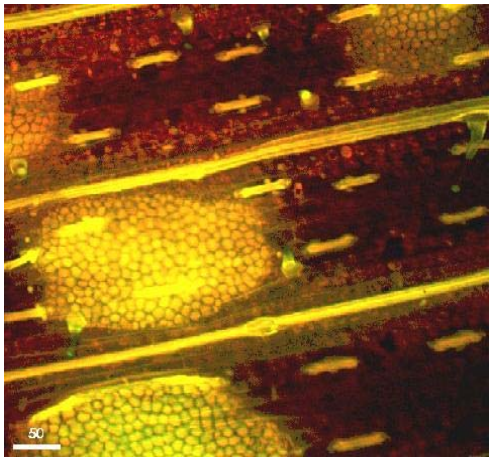

Figure 4. Low intensity of calcium fluorescence with high amount of spores in the infected area of the susceptible genotype, 18 days after inoculation.

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-drived infection of the cowpea rust

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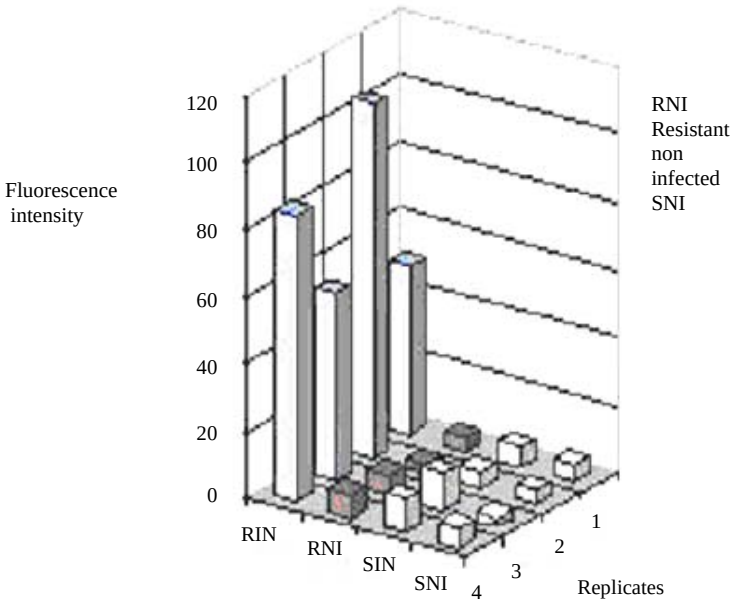

Figure 5. Mean of calcium fluorescence intensity measurements of four replicates by CLSM, using X-Rodamin-1 probe, in infected and non infected areas of the susceptible spring wheat (Thatcher) and its resistant near isogenic line (having *Yr*-18 gene) to yellow rust fungus (*Puccinia striiformis*). Mobilization of calcium (cytosolic free calcium) at the point of challenge by the pathogen, conditioned by the *Yr*-18 gene, was high and played a role of hypersensitive defense response (HR) in infected area of the resistant genotype= RIN). The intensity of calcium fluorescence in the non- infected areas of the resistant near isogenic line (RNI) and in infected and non infected areas of a susceptible host (SIN and SNI) were nearly the same.

fungus. Their calcium levels ratio analysis and imaging studies demonstrated a consistent increase of [ca2+] 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; Tavemier *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 [ca2+] 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 ژن *Yr*-18، عامل ایجاد مقاومت پایدار درنیرایزوژنیک لاین گندم بهاره نسبت به عامل بیماری زنگ نواری با استفاده از میکروسکوپ اسکن لیزری

س. ع. الهی نیا و جی. پی. تواری

چکیده

میکروسکپ لیزری (Confocal Lazer Scanning Microscopy = CLSM) تحولی پیشرفته در زمینه میکروسکپ نوری می باشد که درچند سال اخیر درزمینه مطالعه اثرات متقابل بین بیمارگر و گیاه میزبان مورد بهره برداری قرار گرفته است . برای انجام این مطالعه سوسپانسیون یوردینیوسپور از ایزوله -SR-99 UA(نژاد 128 E 70) در روغن معدنی Soltrol,170 روی برگهای پرچم (flag leaves) گندم بهاره به نام تاچر (Thatcher) و ایزوژنیک لاین (near isogenic line) حاوی ژن مقاوم *Yr*-18 مایه زنی گردید. از برگهای تلقیح شده در روزهای ۲، ۸، ۱۲و۱۸ بعد از مایه زنی نمونه گیری به عمل آمد. برای انجام مشاهده از دو نوع رنگ فلورسنس به نامهای X-Rodamin-1 وAcridin orange استفاده گردید. CLSM این امکان را فراهم ساخت تا با تهیه تصاویر سه بعدی سریال به عمق ۲ میکرون از سطح فوقانی تا سطح تحتانی برگ بدون انجام برش، اثرات متقابل بین بیمارگر و گیاه میزبان مشاهده گردد. نتایج حاصله از مشاهدات نشان داد که جوانه زدن اسپور و نفوذ بیمارگر در دو ژنوتیپ حساس و مقاوم اختلاف قابل توجهی نداشتند. در میزبان حساس فقط تعداد معدودی سلول نکروتیک در ۱۲–۱۸ روز بعد از مایه زنی مشاهده شدند در حالی که در ژنوتیپ مقاوم حاوی ژن *Yr*-18 وسعت بافت مردگی ناشی از واکنش فوق حساسیت (hypersensitive response) قابل توجه بود. میزبان حساس بعد از ۱۸ روز مایه زنی تاول‌های زیاد، با اسپورهای فراوان تولید نمود که این با میزان حساسیت سلولهای میزبان ارتباط مستقیم داشت. در داخل واطراف سلولهای مرده و تاول‌های زنگ در ایزوژنیک لاین حاوی ژن *Yr*-18 کلسیم فلورسنس به میزان قابل توجهی مشاهده گردید در حالی که میزان کلسیم در نواحی غیر آلوده دو ژنوتیپ حساس ومقاوم و همچنین در نواحی آلوده میزبان حساس تقریبا" مشابه بود. این نتیجه نشان داد که انتقال کلسیم از سلول‌های سالم به نواحی مورد حمله بیمارگر (Calcium mobilization) ناشی از اثر ژن*Yr*-18 درژنوتیپ مقاوم بوده است. این اولین گزارش ازاثر انتقال کلسیم درایجاد مقاومت در غلات آلوده به زنگ می باشد.

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