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Research Article

Comparison of ethylene carbonate and formamide as components of the hybridization mixture in FISH

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ABSTRACT: The protocols for *in situ* hybridization (ISH) techniques can vary considerably; however, they usually include denaturation and hybridization steps. Denaturing compounds are used to reduce denaturation and hybridization temperature, which keeps the proper morphology of the preparation. Formamide is the most commonly used reagent in *in situ* hybridization to lower the melting temperature. The substitution of toxic formamide for a non-toxic ethylene carbonate at 20 % and 50 % concentration in the hybridization mixture helped obtain a high quality *in situ* hybridization result with two sequences characteristic for rye, *JNK*, and *Bilby*. The results after hybridization, with a duration of 90 min and 16 h, were identical when formamide or ethylene carbonate were used in the mixture. In addition, the toxic formamide was eliminated from the post-hybridization steps and specific hybridization signals for both probes were still obtained.

Keywords: Secale, *in situ* hybridization, repeated sequences, non-toxic

Introduction

In standard hybridization protocols, denaturation is a very important step that allows generation of single-stranded nucleic acid molecules; thereby, enabling annealing of the probe to the target DNA. High temperature is the simplest denaturation method; however, it can negatively affect the chromosome/tissue morphology. Denaturation of the examined DNA is most often performed with the hybridization solution containing a chemical denaturant, which allows lowering the temperature (Eberwine et al., 1994).

Formamide is a compound known for its ability to lower the melting temperature of DNA (Tm) (Wang et al., 2014), because it has a destabilizing effect on the DNA double helix. The use of formamide in hybridization mixtures increases the hybridization stringency, reducing the background signal (Fuchs et al., 2010; Kessler, 2012). Thus, formamide is often considered crucial for hybridization (Hutton, 1977; Lichter and Cremer, 1992). Unfortunately, formamide is a very dangerous chemical, which may cause carcinogenesis and fertility disorders (Fail et al., 1998; George et al., 2000; George et al., 2002; Sinigaglia et al., 2018). The negative effects of formamide lead to search for less toxic equivalents, while ensuring its advantages in the hybridization process (Durm et al., 1998; Matthiesen and Hansen., 2012).

Compounds, such as urea, sulfolane, gamma-butyryloactone, ethylene carbonate, D-2-pyrrolidone, and γ-valerolactone, were tested in various concentrations (Fontenete et al., 2016; Matthiesen and Hansen, 2012; Sinigaglia et al., 2018). Of all reagents tested, ethylene carbonate proved to be the most optimal for the ISH process and its application reduced hybridization time, denaturation temperature, and background noise.

In this work, fluorescence *in situ* hybridization (FISH) was carried out using two different reagents: formamide and ethylene carbonate at concentrations 20 % and 50 %. Rye, *Secale vavilovii*, was the experimental material. Two molecular probes were used to locate sequences characteristic of the genus of *Secale*: *JNK* and *Bilby*. This study compared the results of FISH analyses using a hybridization mixture containing either formamide or ethylene carbonate at 90 min and 16 h hybridization times. The study also intended to demonstrate whether the replacement of toxic formamide for non-toxic ethylene carbonate affected the intensity and quality of hybridization signals.

Materials and Methods

Plant material

We performed studies with two inbred lines of *Secale vavilovii* Grossh. (109 and 52) obtained by self-pollination of plants, whose anthers and caryopses were mosaic colored, and in 2R chromosomes additional heterochromatin band was present (Achrem et al., 2010; Rogalska et al., 2002).

Fluorescence *in situ* hybridization (FISH)

Chromosome preparations for FISH were made according to the methods of Kalinka and Achrem (2015). DNA was isolated from 1 g of freshly collected rye coleoptiles. The JNK and Bilby probes were both labelled using the PCR method. *JNK* and *Bilby* primers were designed (PRIMER3 software; Rozen and Skaletsky, 2000) using a sequence from *S. cereale* deposited in GenBank (AB008922.1 and AF245032). A pair of primers for JNK probes JNKA: CACAGACCTTGGAATCG TGA and JNKB: TCCGAGTTCGTATGCAAAAGT and Bilby probes bil1: 5′ACTTAGGCGACAAGCCAAGA3′ and bil2: 5′TGTAGCTCATCGTGGAGTCG3′. All primers were synthesized in the IBB Laboratory of DNA Sequencing and Synthesis in Warsaw. *JNK* belongs to

tandem repeat sequences, with a motif length of 1200 bp, showing high similarity to the 5S rDNA fragment and *Angela* retrotransposon (Achrem and Kalinka, 2017). It was located on the long arm of chromosome 2R in the Japanese *S. cereale* cultivar and *S. vavilovii* inbred lines repeated 4,000 times (Nagaki et al., 1999; Rogalska et al., 2002). A 3.4-kb *Bilby* sequence (pAWRC.1), present in the centromeres of all rye chromosomes, was also located (Francki, 2001), although the range of this retrotransposon sequence in some species was extended to the pericentromeric region (Kalinka and Achrem, 2018). PCR labelling for JNK probe was carried out in 25 µL, containing 20 ng of *S. vavilovii* total DNA, 1.2 µM each oligonucleotide primer, 240 µM dATP, dCTP, and dGTP, 150 µM dTTP, 40 µM biotin-11-dUTP, a 1 × buffer, 3.5 mM MgCl2, 4 µg of bovine serum albumin (BSA), and 5 U *Taq* DNA polymerase (native). The PCR conditions were: initial denaturation at 95 °C for 5 min, followed by 30 cycles at 95 °C for 1 min, 48 °C for 1 min, and 72 °C for 2 min, and a final extension at 72 °C for 10 min. *Bilby* reactions were carried out in a 50 µL reaction mixture which contained: 50 ng DNA, 1 × buffer, 2 mM $MgCl_2$, 0.2 mM dATP, dCTP, and dGTP, 0.13 mM dTTP, 40 µM biotin-11-dUTP, 1 µM each primer, 8 µg of bovine serum albumin (BSA), 5 U polymerase. The PCR conditions were: initial denaturation at 95 °C for 5 min, followed by 30 cycles at 95 °C for 1 min, 50.5 °C for 1 min, and 72 °C for 5 min, and a final extension at 72 °C for 5 min.

FISH was performed according to published protocols (Kalinka and Achrem, 2015) with some modifications. The probes were mixed with a hybridization mixture containing: 10 % (m/v) dextran sulfate, 0.1 % (m/v) sodium dodecylsulphate, a buffer of 2 × SSC, and 6 µg mL^{-1} salmon sperm DNA. Used mixtures of hybridization differed by the presence and concentration of ethylene carbonate (20 % or 50 %) or formamide (20 % or 50 %). The chromosomes and probe together were denatured on a hot plate at 83 °C for 5 min. Then, the slides were incubated in a moist chamber at 37 °C for 90 min or 16 h respectively. Post-hybridization washing ensuring high stringency was carried out in 1 × SSC for 4 × 5 min at 42 °C. The slides were incubated in a DB buffer (4 × SSC, 0.2 %, v/v, Tween 20) at 37 °C for 5 min. For signal detection slides were treated with 5 % BSA for 1 h, incubated with avidin-FITC (5 µg mL^{-1}) and anti-avidin antibody (5 µg mL^{-1}). Signal amplification was repeated once. After incubation, the slides were washed three times in a DB buffer at 37 °C for 5 min. The slides were counterstained for 15 min with 1 µg mL^{-1} DAPI in McIlvaine buffer (9 mM citric acid, 80 mM Na2HPO4·H2O, 2.5 mM MgCl2 (pH 7.0)). All slides were mounted in antifade solution (20 mM TrisHCl pH 8.0; 90 % glycerol; 2.3 % DABCO). The preparations were analyzed with an epifluorescence microscope Axio Imager Z2. The resulting images were captured and analyzed using the GENASIs software.

Results and Discussion

Over the years, numerous *in situ* hybridization protocols have been developed and adapted to specific needs, such as the detection of repetitive DNA sequences, short DNA sequences, whole genomes, non-coding RNA or mRNA (Sinigaglia et al., 2018). Most of them recommend using formamide (FA) as a solvent.

Trends in modern science strive for the elimination of toxic substances used during experiments, shortening the test procedure, and development of easy-to-use, yet repeatable protocols. The scientific field is constantly looking for a substance to replace formamide during *in situ* hybridization and that can provide a comparable result. Ethylene carbonate (EC) appears to be a promising alternative to formamide. EC was mainly tested in procedures for animal/human molecular cytogenetics (Matthiesen and Hansen, 2012; Shigeto et al., 2016). However, successful results in these systems may not be consistent with experiments on plant material. The presence of a cell wall significantly impedes *in situ* procedures. The preparation technique of plant chromosomes for molecular cytogenetics differs significantly from preparation of animal/human chromosomes. It is very difficult to remove the cytoplasm and cell wall debris from plant chromosome spreads. Half of the success in FISH results depends on a good quality preparation, and obtaining it is a considerable challenge.

Here, we compared the influence of different concentrations of ethylene carbonate or formamide in a hybridization mixture with the results of FISH on rye (*Secale vavilovii*) chromosomes. Two different times (90 min or 16 h) of hybridization were tested. Probes complementary to the rye repetitive sequences (*JNK* and *Bilby*) were used in this analysis.

The results obtained after *in situ* hybridization in a mixture containing EC were very similar to results of hybridization performed under analogous conditions; however, using a mixture containing FA. Clear hybridization signals were obtained for both probes in which 20 % or 50 % FA/EC were used (Figure 1). Hybridization time did not affect the result. Regardless of whether the hybridization was carried out for 16 h or 90 min, distinct hybridization signals were visible in the chromosomes and cell nuclei. Each of the method modifications obtained specific signals in the centromeric region of all rye chromosomes (*Bilby* probe) or on 2RL chromosomes (*JNK* probe), according to the *Bilby* (Francki, 2001) and *JNK* (Achrem et al., 2010) sequences localization in *Secale vavilovii* chromosomes.

Golczyk (2019) was the first to present the potential use of ethylene carbonate instead of formamide in a FISH procedure for plant material (*Allium*, *Nigella*, *Tradescantia*, *Vicia*). The author used probes designed for repetitive sequences (rDNA) and proved that a hybridization mixture with 15 % EC enables omission of the denaturation step. In this study, ND-FISH (non-

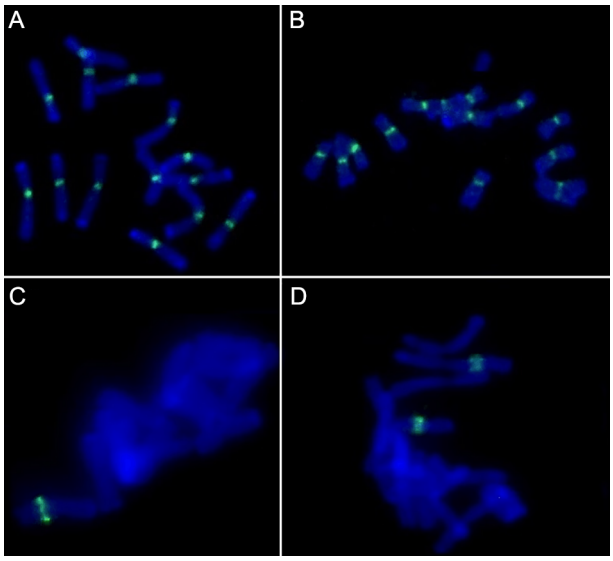

Figure 1 – Fluorescent *in situ* hybridization in *S. vavilovii* with Bilby (A-B) and *JNK* (C-D) probes. (A) 20 % EC, ISH time – 16 h; (B) 20 % FA, ISH time – 16 h; (C) 50 % EC, ISH time – 16 h; (D) 50 % EC, ISH time – 90 min.

denaturing fluorescence *in situ* hybridization) was not tested, because denaturation at 83 °C does not affect chromosome morphology. Golczyk (2019) obtained satisfactory results after hybridization for 3 h. The use of *JNK* and *Bilby* probes allowed to obtain positive hybridization results after 90 min of its duration. Thus, it seems that EC-FISH does not only enable avoidance of toxic compounds, but it may also be a time-saving procedure. However, it should be noted that only repetitive DNA probes were tested and positive results were obtained on the high-quality preparations. Golczyk (2019) analyzed the results of hybridization carried out at two different temperatures (46 °C or 50 °C) in the presence of 15 % EC in the hybridization mixture. The authors also tested two different temperatures (50 °C or 55 °C) of post-hybridization washes in a 2 × SSC solution. All modifications showed clear hybridization signals. The hybridization and post-hybridization conditions were different from those in our protocol, indicating that the EC-method is flexible and can be adapted to various research profiles. Both hybridization and post-hybridization parameters should be determined experimentally.

The results presented in this study confirm the results of EC-FISH analysis of human/animal genomes. Matthiesen and Hansen (2012) found that a short hybridization time (90 min) was definitely sufficient to obtain high quality signals when testing repetitive DNA sequences. Tafe et al. (2015) obtained similar results. The study also used the PNA (Peptide Nucleic Acid) probe complementary to the centromeric region of chromosome 17 (CEN-17). The application of ethylene carbonate shortened the hybridization time and did not require the use of blocking DNA. In addition, it was possible to use a lower denaturation temperature (67 °C), which reduced the background signal (Tafe et al., 2015).

Attempts were also made to eliminate formamide from the FISH procedure without using a substitute. Celeda et al. (1992) proved that repetitive DNA sequences could be detected in the absence of denaturing agents, including formamide. Similar formamide-free FISH procedures were later used in humans (Durm et al., 1996; Celeda et al., 1994; Haar et al., 1994) and plants (Kato et al., 2004; Chester et al., 2012; Jang and Weiss-Schneeweiss, 2015). It should be emphasized that most probes used were complementary to repetitive DNA sequences in these studies. Moreover, in all these protocols, the denaturation step was carried out at high temperatures, between 94 °C and 100°C, which may have a destructive influence on chromosome morphology. The lack of denaturing agent in the hybridization mixture allows shortening the hybridization step. However, to ensure stringency, this hybridization is usually performed at higher temperatures. For example, Kato et al. (2004) conducted denaturation at 100 °C for 5 min, the hybridization step (probe mixed with 2 × SSC and 1 × TE) at 55 °C overnight and stringent washes in 2 × SSC at 55 °C for 20 min. The protocol suggested by Chester et al. (2012) included denaturation at 82-83 °C for 2.5 min, hybridization (probe mixed with 0.7 × SSC) at 55 °C overnight and stringent washes in 2 × SSC at 55 °C. In both protocols, the hybridization and post-hybridization steps were carried out under similar conditions ensuring high stringency. In the EC-FISH protocol, we were able to conduct hybridization and stringent washes at lower temperatures providing hybridization signal specificity. For some probes, other conditions may be optimal, as reported by Jang and Weiss-Schneeweiss (2015), who performed hybridization with a mixture containing 0.02 × SSC at 37 °C and post-hybridization washes in 2 × SSC at 42 °C.

The great advantage of formamide-free FISH protocols is that they reduce the hybridization time; hence, they are often referred to as Fast-FISH. Cuadrado et al. (2009) showed that, regardless of whether hybridization lasted overnight, 2 h, 1 h, or 30 min, they were able to obtain clear hybridization signals. Based on literature data (Matthiesen and Hansen, 2012; Golczyk, 2019) and the results of our work, it seems that the hybridization time may also be reduced in the case of EC-FISH.

Conclusion

This work shows that it is possible to use ethylene carbonate as a non-toxic formamide substitution. Using a buffer containing EC at a concentration of 20 % and incubating for 90 min with stringent post-hybridization washes (1 × SSC, 42 °C) was sufficient to obtain distinct, specific hybridization signals when testing

tandem repeats in the interphase nuclei and metaphase chromosomes of rye. This method follows the trend to simplify and shorten *in situ* hybridization procedures, reducing the risk of researcher exposure to toxins and minimizing toxic waste production (Volpi, 2017).

Authors' Contributions

Conceptualization: Kalinka, A. **Data acquisition:** Kalinka, A.; Myśliwy, M.; Achrem, M. **Data analysis:** Kalinka, A.; Myśliwy, M.; Achrem, M. **Design of methodology:** Kalinka, A.; Myśliwy, M.; Achrem, M. **Writing and editing:** Kalinka, A.; Achrem, M.

References

Achrem, M.; Kalinka, A. 2017. Tracking of intercalary DNA sequences integrated into tandem repeat arrays in *Secale vavilovii*. *Acta Societatis Botanicorum Poloniae 86: 35-48.*

Achrem, M.; Rogalska, S.M.; Kalinka, A. 2010. Possible ancient origin of heterochromatic JNK sequences in chromosomes 2R of *Secale vavilovii* Grossh. *Journal of Applied Genetics* 51: 1-8.

Celeda, D.; Aldinger, K.; Hanr, F.M.; Hausmann, M.; Durm, M.; Ludwig, H.; Cremer, C. 1994. Rapid fluorescence *in situ* hybridization with repetitive DNA probes: quantification by digital image analysis. Cytometry 17: 12-25.

Celeda, D.; Bettag, U.; Cremer, C. 1992. A simplified combination of DNA probe preparation and fluorescence in situ hybridization. Zeitschrift für Naturforschung. Section C 47: 739-47.

Chester, M.; Gallagher, J.P.; Symonds, V.V.; Silva, A.V.C.; Mavrodiev, E.V; Leitch, A.R.; Soltis, P.S.; Soltis, D.E. 2012. Extensive chromosomal variation in a recently formed natural allopolyploid species, *Tragopogon miscellus* (Asteraceae). Proceedings of the National Academy of Sciences of the United States of America 109: 1176–1181.

Cuadrado, A.; Golczyk, H.; Jouve, N. 2009. A novel, simple and rapid nondenaturing FISH (ND-FISH) technique for the detection of plant telomeres: potential used and possible target structures detected. Chromosome Research 17: 755-762.

Durm, M.; Haar, F.M.; Hausmann, M.; Ludwig, H.; Cremer, C. 1996. Optimization of fast-fluorescence in situ hybridization with repetitive alpha-satellite probes. Zeitschrift für Naturforschung C 51: 253-61.

Durm, M.; Sorokine-Durm, I.; Haar, F.M.; Hausmann, M.; Ludwig, H.; Voisin, P.; Cremer, C. 1998. Fast-FISH technique for rapid, simultaneous labeling of all human centromeres. Cytometry 31: 53–162.

Eberwine, J.H.; Valentino, K.L.; Barchas, J.D. 1994. *In Situ* Hybridization in Neurobiology: Advances in Methodology. Oxford University Press, New York, NY, USA.

Fail, P.A.; George, J.D.; Grizzle, T.B.; Heindel, J.J. 1998. Formamide and dimethylformamide: reproductive assessment by continuous breeding in mice. Reproductive Toxicology 12: 317–332.

Fontenete, S.; Carvalho, D.; Guimarães, N.; Madureira, P.; Figueiredo, C.; Wengel, J.; Azevedo, N.F. 2016. Application of locked nucleic acid-based probes in fluorescence *in situ* hybridization. Applied Microbiology and Biotechnology 100: 5897–5906.

Francki, M.G. 2001. Identification of *Bilby*, a diverged centromeric *Ty1-copia* retrotransposon family from cereal rye (*Secale cereale* L.). Genome 44: 266–274.

Fuchs, J.; Dell'Atti, D.; Buhot, A.; Calemczuk, R.; Mascini, M.; Livache, T. 2010. Effects of formamide on the thermal stability of DNA duplexes on biochips. Analytical Biochemistry 397: 132-4.

George, J.D.; Price, C.J.; Marr, M.C.; Myers, C.B.; Jahnke, G.D. 2000. Evaluation of the developmental toxicity of formamide in Sprague-Dawley (CD) rats. Toxicological Sciences 57: 284-291.

George, J.D.; Price, C.J.; Marr, M.C.; Myers, C.B.; Jahnke, G.D. 2002. Evaluation of the developmental toxicity of formamide in New Zealand white rabbits. Toxicological Sciences 69: 165–74.

Golczyk, H. 2019. A simple non-toxic ethylene carbonate fluorescence *in situ* hybridization (EC-FISH) for simultaneous detection of repetitive DNA sequences and fluorescent bands in plants. Protoplasma 256: 873–880.

Haar, F.M.; Durm, M.; Aldinger, K.; Celeda, D.; Hausmann, M.; Ludwig, H.; Crèmer, C. 1994. A rapid FISH technique for quantitative microscopy. BioTechniques 17: 346-353.

Hutton, J.R. 1977. Renaturation kinetics and thermal stability of DNA in aqueous solutions of formamide and urea. Nucleic Acids Research 4: 3537-3555.

Jang, T-S.; Weiss-Schneeweiss, H. 2015. Formamide-free genomic in situ hybridization (ff-GISH) allows unambiguous discrimination of highly similar parental genomes in diploid hybrids and allopolyploids. Cytogenetic Genome Research 146: 325–331.

Kalinka, A.; Achrem, M. 2015. Analysis of the flanking sequences of the heterochromatic JNK region in *Secale vavilovii* Grossh. chromosomes. Biologia Plantarum 59: 637-644.

Kalinka, A.; Achrem, M. 2018. Reorganization of wheat and rye genomes in octoploid triticale (× *Triticosecale*). Planta 247: 807-829.

Kato, A.; Lamb, J.C.; Birchler, J.A. 2004. Chromosome painting using repetitive DNA sequence as probes for somatic chromosome identification in maize. Proceedings of the National Academy of Sciences of the United States of America 101: 13554–13559.

Kessler, C. 2012. Overview of nonradioactive labeling systems. p. 27-34. In: Kessler, C., ed. Nonradioactive labeling and detection of biomolecules. Springer, Berlin, Germany.

Lichter, P.; Cremer, T. 1992. Chromosome analysis by non-isotopic *in situ* hybridization. p. 157-192. In: Rooney, D.E.; Czepulkowski, B.H., eds. Human cytogenetics: a practical approach. 2ed. IRL Press, Oxford, UK.

Matthiesen, S.H.; Hansen, C.M. 2012. Fast and non-toxic in situ hybridization without blocking of repetitive sequences. PLoS One 7: e40675.

Nagaki, K.; Tsujimoto, H.; Saskuma, T. 1999. A novel repetitive sequence, termed the JNK repeat family, located on an extra heterochromatic region of chromosome 2R of Japanese rye. Chromosome Research 6: 95-101.

Rogalska, S.M.; Achrem, M.; Słomińska-Walkowiak, R.; Filip, E.; Skuza, L.; Pawłowska, J.; Apolinarska, B. 2002. Polymorphism of heterochromatin bands on chromosomes of rye *Secale vavilovii* Grossh. lines. Acta Biologica Cracoviensia series Botanica 44: 111-117.

Rozen, S.; Skaletsky, H.J. 2000. Primer3 on the WWW for general users and for biologist programmers. Methods in Molecular Biology 132: 365-386.

Shigeto, S.; Matsuda, K.; Yamaguchi, A.; Sueki, A.; Uehara, M.; Sugano, M.; Uehara, T.; Honda, T. 2016. Rapid diagnosis of acute promyelocytic leukemia with the PML-RARA fusion gene using a combination of droplet-reverse transcription-polymerase chain reaction and instantquality fluorescence *in situ* hybridization. Clinica Chimica Acta 453: 38-41.

Sinigaglia, C.; Thiel, D.; Hejnol, A.; Houliston, E.; Leclère, L. 2018. A safer, urea-based *in situ* hybridization method improves detection of gene expression in diverse animal species. Developmental Biology 434: 15-23.

Tafe, L.J.; Steinmetz, H.B.; Allen, S.F.; Dokus, B.J.; Tsongalis, G.J. 2015. Rapid fluorescence *in situ* hybridization (FISH) for HER2 (ERBB2) assessment in breast and gastrooesophageal cancer. *Journal* of *Clinical Pathology* 68: 306-308.

Volpi, E.V. 2017. Formamide-free fluorescence in situ hybridization (FISH). p. 135-139. In: Liehr, T., ed. Fluorescence *in situ* hybridization (FISH). Springer, Berlin, Germany.

Wang, X.; Lim, J.H.; Son, A. 2014. Characterization of denaturation and renaturation of DNA for DNA hybridization. Environmental Health and Toxicology 29: e2014007.