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COMMUNICATION

Arabidopsis thaliana ITS sequence-specific DNA extraction by ion-tagged oligonucleotides coupled with a magnetic ionic liquid

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Abstract

This study reports a follow-up investigation on the capture of specific DNA sequences using ion-tagged oligonucleotides (ITOs) and magnetic ionic liquids (MIL). Five allylimidazolium salts bearing octyl substituents ([AOIM$^+$]-ITOs) were used for the selective extraction of the internal transcribed spacer region (*ITS*) from *Arabidopsis thaliana*. In this work, the ability of the [AOIM$^+$]-ITOs to enhance the extraction of longer target sequences (~ 700 bp) of plant origin was shown. Moreover, the independence of the probe binding position and the importance of complementarity to the target region for the extraction performance were demonstrated. To test the specificity of the ITOs, the same experiments were performed using the *ITS* region from another plant species, with a lower target capture for the probes which were specific for the *A. thaliana* sequence. Finally, extraction in the presence of interferences (heterogenous DNA, primary and secondary metabolites, proteins) provided interesting and insightful results. This work illustrates the feasibility and versatility of these probes when coupled to MILs for rapid, cost-effective, and environmentally sensitive sample preparation in the extraction of specific target sequences from different origins.

Keywords Sequence-specific DNA extraction · *Arabidopsis thaliana* · Ion-tagged oligonucleotide · Magnetic ionic liquid · Internal transcribed spacer

Introduction

The importance of nucleic acids as marker molecules is well known. The detection of small DNA sequences in different types of samples (e.g., blood, plant tissues) can facilitate tumor or disease diagnosis (pathogen infections). At the same time, the authentication of an unknown plant/animal specimen can be performed by evaluating the nucleotide variation of specific DNA regions (e.g., DNA barcoding) [1–3]. Therefore, the selective extraction of target sequences is an important issue in molecular biology. Several different methods to capture and enrich specific nucleic acids have been developed and proposed. Probes capable of binding a complementary sequence can be captured by solid supports (e.g., magnetic beads, nanoparticles, microarrays) or liquid supports (e.g., ionic liquids) for the specific extraction of sequences related, for instance, to cancer or pathogen agents [4–9]. Among these techniques, magnetic beads or particles coated with streptavidin are the most popular, despite the possibility of the solid support undergoing aggregation, which can compromise the extraction efficiency and clog liquid handling devices [1]. With this background, a new selective extraction technique using ion-tagged oligonucleotides (ITO) coupled to magnetic ionic liquids (MIL) was developed by Clark and coworkers [10]. The ITO structure consists of an imidazolium-based ion tag linked to an oligonucleotide through thiol-ene click chemistry. The imidazolium-based ion tag strengthens the partitioning of the oligonucleotide to the hydrophobic MIL, while at the same time the complementarity of the oligonucleotide to a specific DNA sequence promotes the

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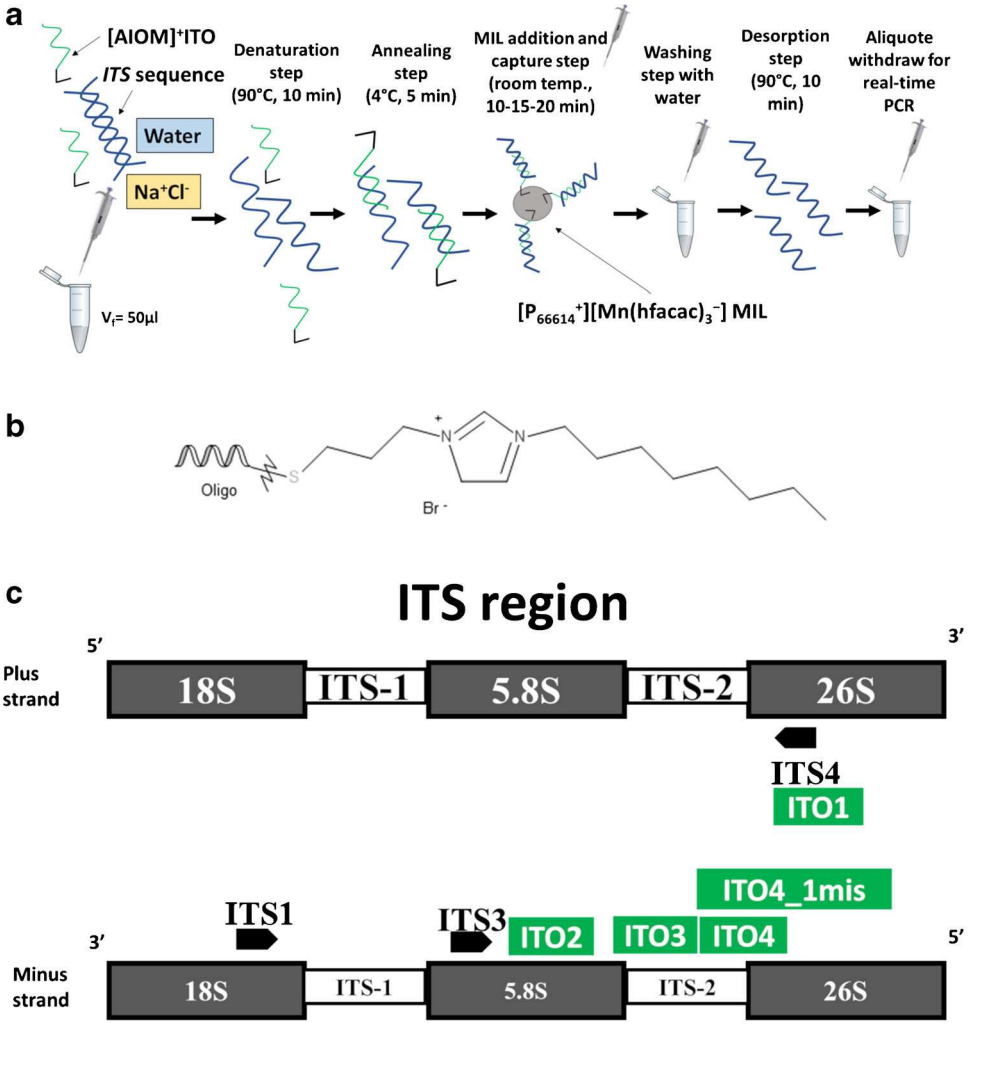
selective extraction of the target of interest. Compared to traditional extraction techniques, the main advantage of this method lies in the liquid nature of the MILs that prevents the formation of aggregates. At the same time, it is a high throughput technique that does not require an agitation step. Moreover, the ITO-MIL strategy exhibits a comparable extraction efficiency to magnetic bead procedures, while not being influenced by the presence of interferences, contrary to the traditional approach [10].

Taking a cue from this new strategy, this study evaluates the extraction of a plant DNA sequence by using allylimidazolium salts bearing different octyl substituent ion-tagged oligonucleotides ([AOIM$^+$]-ITOs) coupled with the [P_{66614}^+][Mn(hfacac)$_3^-$] MIL. A schematic of the extraction process used in this work is shown in Fig. 1a. The [AOIM$^+$]-ITO (Fig. 1b) was chosen for its optimal partitioning capacity to the [P_{66614}^+][Mn(hfacac)$_3^-$] MIL [10].

The aim of this work is to develop a proof of concept study to test the feasibility and versatility of the ITO-MIL method by increasing the complexity of the sample under investigation as well as extend the number and type of application fields (e.g., plant metabarcoding, plant pathogen genes, and GMO



d

3' thiolated ITO	Nucleotide sequence
ITO1	5'-TCCTCCGCTTATTGATATGC/C3SH-3'
ITO2	5'-AAATGCGATACTTGGTGTGA/C3SH -3'
ITO3	5'-GTACCGACATGCGGTGGTGA/C3SH -3'
ITO4	5'-TAGATGACCCAAAGTCCATATAGCG/C3SH-3'
ITO4_1mis	5'-TAGATGACCCAA**G**GTCCATATAGCG/C3SH-3'

Fig. 1 (**a**) Schematic representation of the [AOIM$^+$]-ITO-MIL extraction strategy. (**b**) Scheme of the [AOIM$^+$]-ITO structure. (**c**) Structure of the internal transcribed spacer; arrows indicate the position of the primers (ITS1, ITS3, ITS4), squares indicate the position of the [AOIM$^+$]-ITOs (ITO1, ITO2, ITO3, ITO4, ITO4_1mis). (**d**) 3'thiolated ITO nucleotide sequences

detection) [7, 11–13]. With this purpose, *Arabidopsis thaliana* (L.) Heynh and the internal transcribed spacer (*ITS*) region were chosen since they are considered as a model plant and a model marker gene, respectively [2, 14, 15].The sequence selected in this work was isolated directly from a plant organism and is longer (~ 700 bp) compared to the 261 bp synthetized target sequence that was previously examined by Clark et al. [10]. Various ITOs (containing different sequences) were used to extract the *A. thaliana ITS* sequence from an aqueous solution. Concurrently, the specificity of the [AOIM$^+$]-ITOs was tested in the extraction of the same *ITS* region from a different plant species (*Ptilostemon casabonae* (L.) Greuter) or in the presence of interferences (heterogenous DNA, primary and secondary metabolites, proteins). The *ITS* sequence is divided into different sections, namely, the rDNA transcription units (18S, 5.8S, and 26S) which are more conserved among different plant species and the more variable ITS-1 and ITS-2 regions (Fig. 1c). The ITOs used in this work were selected to bind *ITS* in both transcription and spacer units that are located in the far end and middle of the sequence, while in the previous study, the probe was fully complementary to the terminal segment of the sequence of interest [10]. Three 20-mer ITOs with different features were designed. Among them, ITO1 corresponds to the *ITS4* plant universal primer [16], and lacks full complementarity resulting in binding of the *ITS* sequence at the 3′ end. Two additional probes were selected, with one binding to the 5.8 s rDNA transcription unit and being fully complementary to both the *A. thaliana* and *P. casabonae ITS* sequences (ITO2) and the other probe binding the *ITS2* region specific for the *A. thaliana ITS* region and not *P. casabonae* (ITO3). Moreover, for the first time, a longer probe of 25-mer (instead of 20-mer), complementary to the *ITS2* region, was synthetized and tested (ITO4) [10]. Finally, the ITO4_1mis with the same nucleotide composition as ITO4 but with one nucleotide mismatch was designed.

The *A. thaliana* (U43225.1) and *P. casabonae* (MK005004) *ITS* sequences deposited in GenBank were used as template for the [AOIM$^+$]-ITO design (see Electronic Supplementary Material (ESM) Fig. S1a, b).

Materials and methods

Reagents and materials

All chemicals were obtained from Sigma Aldrich (St. Louis, MO, USA) unless otherwise indicated. Tris(hydroxymethyl)aminomethane, urea, boric acid, and tris(2-carboxyethyl) phosphine (TCEP) were purchased from P212121 (Ypsilanti, MI, USA). LCMS-grade water was obtained from Fisher Scientific (Fair Lawn, NJ, USA). Dimethyl sulfoxide (DMSO) was obtained from Fisher Scientific (Fair Lawn, NJ, USA). Ammonium persulfate (APS), tetramethylethylenediamine (TEMED), 40% acrylamide, and bis-acrylamide solution 29:1, SsoAdvanced Universal SYBR Green Supermix, were purchased from Bio-Rad Laboratories (Hercules, CA, USA). A Milli-Q water purification system was used to obtain deionized water (18.2 MΩ cm) for the preparation of all solutions (Millipore, Bedford, MA, USA). Thiolated oligonucleotides and primers were purchased from IDT (Coralville, IA, USA).

The [P_{66614}^+][Mn(hfacac)$_3^-$] MIL and the [AOIM$^+$][Br$^-$] investigated in this study were synthesized and characterized according to previously reported procedures [10, 17]. The 3′ thiol-modified oligonucleotide sequences used in this study are reported in Fig. 1d.

ITO synthesis and analysis

For [AOIM$^+$]-ITO synthesis, 1 μL of thiolated oligonucleotide (4 nmol/μL) was added to 0.4 μL of TCEP. After 15 min at room temperature, 0.5 μl of [AOIM$^+$][Br$^-$] were added in a Greiner 96-well half area, UV-star clear microplate (Kremsmünster, Austria), and the well was filled with a stream of nitrogen, covered and incubated under a UVGL-58 handheld lamp from UVP (Upland, CA, USA). A NotePal X-slim Cooler Master (New Taipei City, Taiwan) was used to cool the well plate.

An 18% (*v*/*w*) polyacrylamide gel was prepared with 7 M urea to resolve the ITOs from the unreacted oligonucleotides. The gel was run at 200 V and 150 W for 1.5 h with an ice bath to cool the electrophoresis tank (Mini Protean 3 electrophoresis system from Bio-Rad Laboratories with an ECPS 3000/150 power supply from Pharmacia; Stockholm, Sweden). An Agilent 1260 HPLC with a diode array detector coupled to an Agilent 6230B Accurate Mass time-of-flight (TOF) mass spectrometer with an electrospray source was employed for the ITO characterization. The separation of the ITOs was performed on an Agilent Technologies 50 mm × 2.1 mm i.d. × 1.8 μm particle Zorbax Extend-C18 column. The column was equilibrated for 9 min at 0.2 mL min^{-1} with a mobile phase composition of 95:5 A:B, using 5 mM triethylammonium acetate (pH 7.4) and acetonitrile as mobile phases A and B, respectively. Gradient elution was performed with the following program: 5% B from 0 to 3 min, increase to 19.4% B from 3 to 7 min, increase to 35% B from 7 to 8 min, hold at 35% B from 8 to 10 min, increased to 100% from 10 to 20 min, hold at 100% from 20 to 23, decreased to 5% B from 23 to 27 min. The LC eluent was diverted to waste for the first 8 min to prevent non-volatile imidazolium salts from the reaction mixture from entering the mass spectrometer. The nebulizing gas was set to 35 psi. The drying (N_2) gas flow rate was 9 L min^{-1} with a temperature of 350 °C. The capillary voltage was 4000 V. Spectra were acquired in the range 100–3000 *m*/*z* with a scan rate of 1 spectrum sec^{-1}.

Plant DNA samples

Arabidopsis thaliana (L.) Heynh, used for the amplification of the *ITS* region used in this work, was grown at the Department of Life Sciences and Systems Biology (University of Torino) as reported by Marengo et al. 2019 [18] without any modification.

Ptilostemon casabonae (L.) Greuter was collected from Sardinia (Gennargentu; OG, 39° 53′ 54.9″ N–9° 26′ 27.9″ E) and the leaves were dried at room temperature until constant weight and ground with a mortar and pestle to obtain a fine powder [19].

Ten milligrams of dried ground leaves of both *A. thaliana* and *P. casabonae* was employed for DNA extraction using the Nucleospin Plant II Kit (Macherey Nagel, Düren, Germany) following the manufacturer's instructions. Approximately 20 ng of genomic DNA was used as a template for PCR amplification, with forward (ITS1:5′-TCCGTAGGTGAACCTGCGG-3′) and reverse (ITS4: 5′-TCCTCCGCTTATTGATATGC-3′) primers specific for the amplification of the *ITS* region [16]. PCR reactions were carried out in a T-Gradient Thermalcycler (Biometra, Jena, Germany). Amplification was performed with 2.5 μL of 10X PCR buffer (Thermo-Scientific, Waltham, MA, USA), 0.2 mM deoxynucleotide triphosphates (dNTPs) (Thermo-Scientific, Waltham, MA, USA), 20 pmol of forward and reverse primers (Integrated DNA Technologies, BVBA, Leuven, Belgium), and 0.5 U of *Taq* DNA polymerase (Thermo-Scientific, Waltham, MA, USA) to a final volume of 25 μL. Cycling conditions were as follow: 4 min at 94 °C, followed by 30 s denaturing at 94 °C, 45 s annealing, respectively, at 53° and 45 s elongation 72 °C, repeated for 35 cycles and with 5 min final extension at 72 °C. PCR products were separated by 1.5% (*w*/*v*) agarose gel electrophoresis and visualized by ethidium bromide staining under UV [18].

The obtained bands were excised from the gel and purified with a QIAquick gel extraction kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

Each purified PCR product was quantified using a Nanodrop 2000c spectrophotometer (Thermo Fisher Scientific); a standard solution of 2 ng μL^{-1} was then serially diluted and stored at − 20 °C.

Concerning the experiments in which interferences were evaluated, *A. thaliana* genomic DNA was extracted using the Nucleospin Plant II Kit (Macherey Nagel, Düren, Germany) as previously described. *Nicotiana benthamiana* Domin leaves were obtained from the Department of Plant Pathology and Microbiology, Iowa State University, Ames, IA; they were dried at room temperature until constant weight and ground with a mortar and pestle to obtain a fine powder. Five hundred microliters of a buffer solution containing 50 mM Tris-EDTA and 3 μM sodium dodecyl sulfate (SDS) were added to 10 mg of ground *N. benthamiana* leaves and incubated for 15 min at 100 °C to obtain the plant cell lysate. After 15 min of centrifugation at maximum speed, the supernatant was collected and used for the subsequent tests [18].

[AOIM$^+$]-ITO-MIL extraction conditions

The extraction was performed according to Clark and coworkers with some modifications [10]. In a PCR tube, 1.69 pmol of [AOIM$^+$]-ITO and 16.9 fmol of target DNA were added to 25 mM NaCl, water and 5% (*v*/*v*) DMSO when necessary, to a final volume of 50 μL. For the experiments in which interferences were present, water was replaced with 0.7 μg of *A. thaliana* genomic DNA or plant cell lysis from *N. benthamiana*. Hybridization of the [AOIM$^+$]-ITO to target DNA was performed at 90 °C for 10 min, followed by an annealing step for 5 min at 4 °C (50–60–70–75 °C were selected for the experiments with ITO4_1mis). The MIL was then added to the solution, in the middle of the tube, and incubated at room temperature for 10, 15, or 20 min to extract the ITO-target duplex. After decanting the sample solution and washing the MIL twice with water, the target was eluted from the ITO in 50 μL of deionized water at 90 °C for 10 min and an aliquot of 30 μL was stored for further analysis. For every assay, a sample in which the [AOIM$^+$]-ITO was replaced with water was used as negative control.

qPCR conditions

A CFX96 Touch Real-Time PCR Detection System from Bio-Rad Laboratories was used for the real-time quantitative PCR (qPCR) assays. In all reactions, 1 μL of template DNA was added to a 19 μL reaction mix that contained 10 μL of SsoAdvanced Universal SYBR Green Supermix (2X), 8.6 μL of deionized water and 0.4 μL of 10 μM forward and reverse primers. Primers for qPCR amplification of the ITS region were 5′- CACGCTTACATTCAC GCCCT -3′ and 5′- CGAGCGTC CCAAAACCTTCT -3′ [16]. The thermal cycling protocol for all qPCR assays was as follows: an initial denaturation step of 10 min at 95 °C followed by 40 cycles of 15 s at 95 °C and 45 s at 65 °C. A qPCR calibration curve of ITS target of both *A. thaliana* and *P. casabonae* with a ten-fold dilution series was performed (Fig. 2).

Statistical analysis

All experiments were carried out in triplicate. ANOVA analysis was performed using SPSS 15.0 (IBM Corporation) software.

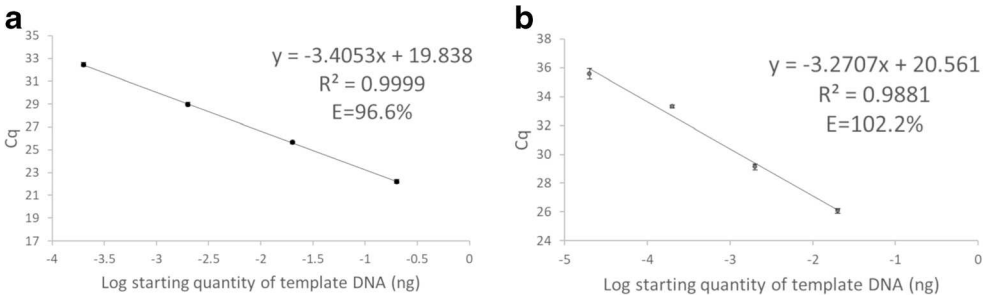


Fig. 2 Ten-fold dilution series qPCR calibration curve of the *ITS* target sequence from *A. thaliana* (**a**) and *P. casabonae* (**b**). Amplification efficiency was calculated from the slope using the eq. $E = (10^{-1/slope})-1$. Cq = quantification cycle

Results and discussion

Extraction of the *ITS* region of A. thaliana with different [AOIM$^+$] ion-tagged oligonucleotides

In the present work, the selective extraction of *A. thaliana ITS* region with different [AOIM$^+$]-ITOs was evaluated. The approach adopted in this work is the same as reported by Clark and co-workers [10], with minor modifications. The evaluation of different extraction times (10, 15, and 20 min) to optimize the extraction method with the [AOIM$^+$]-ITOs used in this work was also performed using similar approaches to those previously published [10]. As shown in Fig. 3a–c, a 10 min extraction was optimal for ITO2, while a 15 min extraction was the best extraction time for ITO3 and ITO4. The quantification cycles (Cq values) indicated an approximate 10-fold higher amount of DNA extracted with the [AOIM$^+$]-ITO-MIL approach at the optimized extraction times, compared to the negative control (Cq values: ITO2➔27.24 Vs. NegCtrl➔30.05; ITO3➔26.60, ITO4➔26.69 Vs. NegCtrl➔29.71). On the other hand, ITO1 did not show a significant enhancement in DNA extraction efficiency and this result could be explained by the lacking full complementarity of the probe (corresponding to the ITS4 plant universal

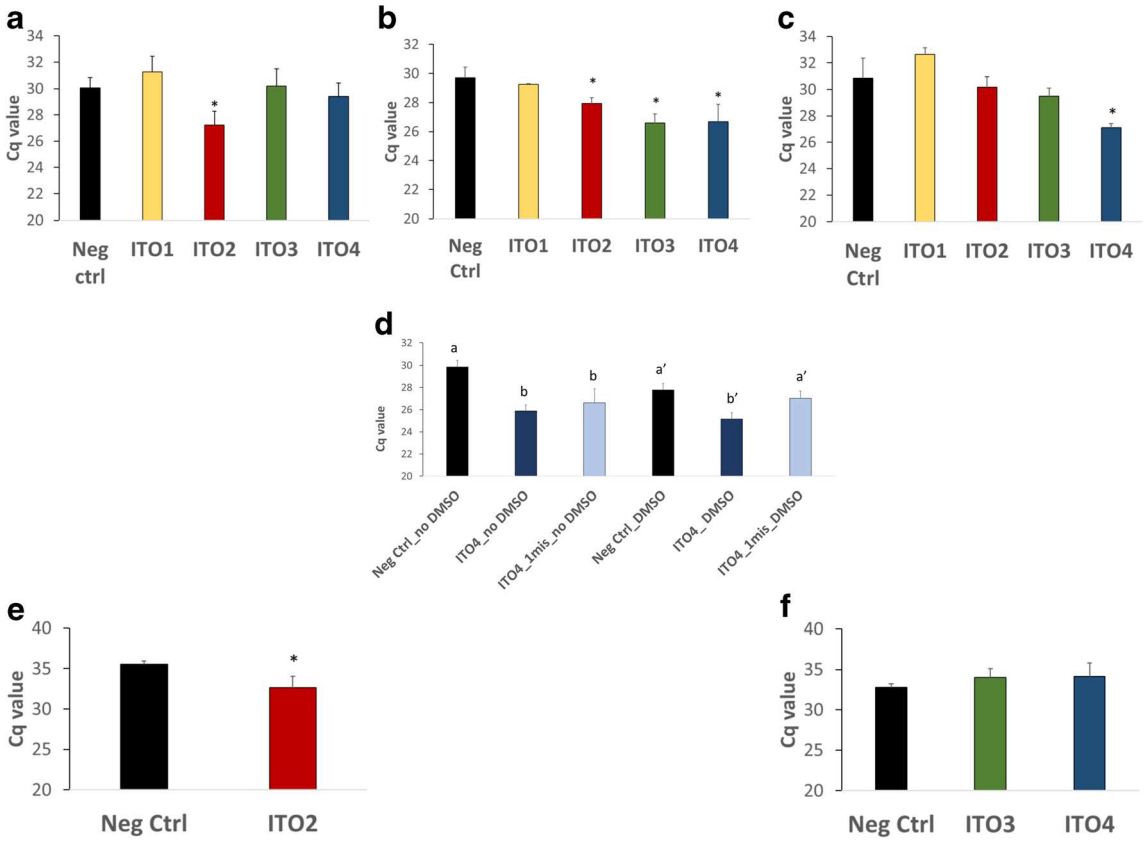


Fig. 3 Quantification cycles (Cq values) of *A. thaliana ITS* extraction performed by ITO1, ITO2 ITO3, ITO4 at different extraction times: 10 min (**a**), 15 min (**b**), 20 min (**c**). (**d**) ITO4-1mis efficiency for the extraction of *A. thaliana* ITS region with or without the addition of DMSO. Quantification cycles (Cq values) of *P. casabonae ITS* extraction performed by ITO2 (10 min extraction) (**e**), ITO3, and ITO4 (15 min extraction) (**f**). Neg Ctrl: negative control, in which the DNA was replaced with water. Asterisks indicate significant differences at $p < 0.05$. Different letters indicate significant differences at $p < 0.05$

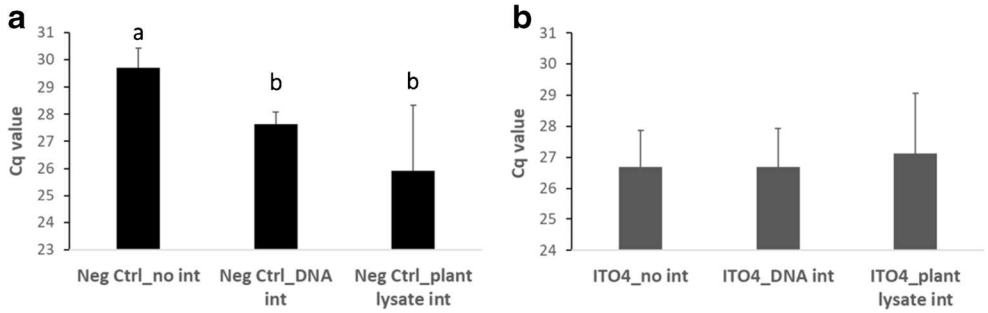


Fig. 4 Quantification cycles (Cq values) of *A. thaliana ITS* extraction performed by the $[P_{66614}^{+}][Mn(hfacac)_3^{-}]$ MIL alone (**a**) or with the addition of the ITO4 (**b**) with or without interferences. No int: without interferences. DNA int: *A thaliana* genomic DNA spike. Plant lysate int: *N. benthamiana* lysate spike. Different letters indicate significant differences at $p < 0.05$

primer) with the *ITS* sequence. For this reason, the interaction between the sequence and the oligonucleotide was likely reduced. To further investigate the specificity of the tested [AOIM$^+$]-ITOs in the extraction of the region of interest, a probe analogous to ITO4 containing one nucleotide mismatch was synthetized (ITO4_1mis). Both ITO4 and ITO4_1mis were used to capture the *A. thaliana ITS* sequence from an aqueous solution and the relative extraction efficiency was compared. As reported in Fig. 3d, both [AOIM$^+$]-ITOs showed a higher extraction efficiency compared to the negative control, but no statistical difference in the ability between ITO4 and ITO4_1mis to extract the *ITS* sequence was observed. The same results were obtained by increasing the specificity of the probe to bind the *ITS* sequence through the use of higher annealing temperatures (50 °C, 60 °C, 70 °C, 75 °C) (see ESM Fig. S2). These findings demonstrated that the one nucleotide mismatch probe exhibited similar extraction efficiency to the sequence of interest compared to the fully complementary probe. This highlights the difficulty in discriminating sequences with a highly similar nucleotide composition. However, the addition of 5% (*v*/*v*) DMSO to the initial solution gave different results at an annealing temperature of 75 °C. Figure 3d shows a significantly higher extraction efficiency of the *ITS* sequence using ITO4 in the presence of DMSO, compared to ITO4_1mis. This result can be explained by the ability of DMSO to decrease the melting temperature [20] (Fig. 3d), as it could be used to enhance the specificity of the probe in the extraction of highly similar nucleotide sequences, such as when single-nucleotide polymorphisms (SNPs) are present.

All the experiments were performed in triplicate with good results in terms of reproducibility, with a relative standard deviation percentage (RSD%) never exceeding 5%.

The present strategy is capable of enhancing the extraction efficiency of the target when the ITO is highly complementary to the sequence of interest. Furthermore, the results suggest that DMSO can be used to further increase the specificity of the probes in the presence of sequences with a very low nucleotide variation. Additionally, it was demonstrated that the ITO binding position does not affect the ability of the probe to selectively extract the *ITS* region as ITO2, ITO3, and ITO4 gave similar results. Finally, probes with different length did not show statistical differences in the selective extraction of the *ITS* region. The obtained results are in agreement with previous literature data, in that the ITOs are able to selectively extract the sequence of interest, with lower extraction efficiencies compared to Clark et al. [10]. The 10-fold extraction efficiency obtained in this work is not comparable to the enrichment previously described, likely because of the difference in the nature of the samples under analysis. However, since the aim of this study is the specific extraction of a target sequence, a 10-fold enhancement is an acceptable result considering the complexity of the sample under investigation.

Extraction of the *ITS* region of *P. casabonae* with different [AOIM$^+$] ion-tagged oligonucleotides

The specificity of the synthetized [AOIM$^+$]-ITOs was further investigated by simulating the extraction of an *ITS* sequence obtained from another plant species (*P. casabonae*). The three [AOIM$^+$]-ITOs fully complementary to the *A. thaliana ITS* region that provided positive results in its extraction were examined. The experiments were performed at the optimal extraction time for each probe. As shown in Fig. 3e, f, ITO2 was able to enhance the extraction efficiency of the *ITS* sequence from *P. casabonae*, while for ITO3 and ITO4, no significant differences were obtained compared to the control. This result can be explained by the fact that ITO2 was complementary to the 5.8 s rDNA transcription unit, which is a highly conserved region of the *ITS* sequence, while ITO3 and ITO4 bind the ITS2 region that vary widely in different species [2]. This is confirmed by the alignment of the oligonucleotide sequences with the *ITS P. casabonae* sequence (MK005004). As expected, ITO2 is fully complementary while ITO3 and ITO4 did not match with the sequence (see ESM Fig. S1b). These findings suggest the possibility of designing ITOs complementary to conserved regions that are able to detect a target sequence from different plant species

or, on the contrary, specific ITOs which bind the sequences in a variable part of the sequence of interest to provide more selectivity.

A. thaliana *ITS* region extraction with ITO4 in the presence of interferences

Since biological samples usually contain high levels of interferences, the effects of interfering heterogeneous sequences and plant cell lysates on the selective extraction of *A. thaliana ITS* sequence were considered.

Starting solutions containing the target DNA were spiked with 0.7 μg of *A. thaliana* genomic DNA used as a heterogeneous sequence or with plant cell lysate from *Nicotiana benthamiana* which contains different types of interferences (primary and secondary metabolites, cell residuals, proteins). These experiments were performed with the aim to reproduce a condition that more closely mimics real samples that are usually handled in common laboratories. The extraction performed with the $[P_{66614}^{+}][Mn(hfacac)_3^{-}]$ MIL alone (Fig. 4a) showed a higher extraction efficiency in the presence of both type of interferences, suggesting a non-selective target extraction. On the other hand, a comparable extraction efficiency was obtained for ITO4 with or without interferences (Fig. 4b). These findings suggest that the specificity of the ITOs is not influenced by the presence of genomic DNA, secondary metabolites, or cell residuals that may affect biomolecular analysis.

Conclusions

This work provides an improvement in the sequence-specific DNA extraction approach that involves ITO probes coupled to MILs. This method resulted in the successful extraction of longer DNA sequences from plants with a picomolar concentration. Moreover, it was demonstrated that the binding position of the probes to the sequence did not influence the extraction efficiency. These findings add relevant information regarding the use of ITO selective extraction coupled with MILs in order to extend its feasibility and evaluate its versatility. In future, this technique has the potential for use in rapid, cost-effective, and environmentally sensitive sample preparation for the extraction of specific target sequences from different origin. Moreover, recent improvements in increasing the loading capacity of the target by improving the ITO design (synthesis of disubstituted ion-tagged oligonucleotide (DTO) [1]) have the potential to provide lower detection limits. Further studies should be conducted with these new probes and on other types of marker sequences from different organisms to extend the full potential of the approach in diagnostic (plant or human pathogens, cancer) and authentication (food adulteration, GMO) fields.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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