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TILLING mutants of durum wheat result in a high amylose phenotype and provide information on alternative splicing mechanisms

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Abstract

The amylose/amylopectin ratio has a major influence over the properties of starch and determines its optimal end use. Here, high amylose durum wheat has been bred by combining knock down alleles at the two homoelogous genes encoding starch branching enzyme IIa (*SBEIIa-A* and *SBEIIa-B*). The complete silencing of these genes had a number of pleiotropic effects on starch synthesis: it affected the transcriptional activity of *SBEIIb*, *ISA1* (starch debranching enzyme) and all of the genes encoding starch synthases (*SSI*, *SSIIa*, *SSIII* and *GBSSI*). The starch produced by grain of the double *SBEIIa* mutants was high in amylose (up to ~1.95 fold that of the wild type) and contained up to about eight fold more resistant starch. A single nucleotide polymorphism adjacent to the splice site at the end of exon 10 of the G364E mutant copies of both *SBEIIa-A* and *SBEIIa-B* resulted in the loss of a conserved exonic splicing silencer element. Its starch was similar to that of the *SBEIIa* double mutant. G364E *SBEIIa* pre-mRNA was incorrectly processed, resulting in the formation of alternative, but non-functional splicing products.

Keywords: alternative splicing, durum wheat, high amylose, mRNA processing, resistant starch, TILLING

Abbreviations

AC, amylose content; DPA, day post anthesis; ESE, exonic splicing enhancer; ESS, exonic splicing silencer; GBSSI, granule bound starch synthase; HGW, hundred grain weight; HRM, high resolution melting; ISA1, 2, 3, isoamylase 1, 2, 3; LD, limit dextrinase; qRT-PCR, quantitative real time-PCR; RS, resistant starch; RT-PCR, reverse transcription-PCR; SBEIIa, IIb, starch branching enzyme IIa, IIb; SSI, II, III, starch synthase I, II, III; TILLING, targeting induced local lesions in genomes; TS, total starch.

1. Introduction

Grain wheat starch comprises a combination of the two glucan polymers amylose and amylopectin. Amylopectin is produced by the concerted action of several classes of starch synthase (SSI, SSII and SSIII), branching (SBEI, SBEIIa, and SBEIIb) and debranching enzymes (isoamylases ISA1, ISA2, ISA3 and limit dextrinase [LD]); in contrast, the sole enzyme involved in amylose synthesis is the granule-bound starch synthase I (GBSSI) [1]. Durum wheat (*Triticum turgidum* subsp. *durum*) is a widely cultivated crop species used for the manufacture of pasta and couscous (along with other similar products in Western Asia and North Africa) and forms a major ingredient in both leavened and unleavened bread throughout the Mediterranean Basin.

The amylose to amylopectin ratio has a major effect on pasta quality [2, 3]. High amylose starch has a higher resistant starch (RS) content; like dietary fibre, its consumption can be beneficial for human health [4-7]. About 25% of wheat starch comprises amylose, but this proportion can be increased by suppressing one or more of the genes – particularly *SSII* and *SBEIIa* - involved in amylopectin synthesis [8-16].

There has been a recent resurgence of interest in mutagenesis, both as a genetic and a breeding tool [17-19]. The TILLING (Targeting Induced Local Lesions IN Genomes) approach combines chemical mutagenesis with DNA-based screening to identify mutations to a specific gene target [20]. First developed in the model species *Arabidopsis thaliana* [20], it has been applied very widely among crop species [21]. Several wheat TILLING projects have been initiated, targeting either agronomic or quality traits [11, 13-15, 22-28]. The study and characterization of loss-of-function and missense mutations can contribute to understand molecular processes not well-established, as the pre-mRNA processing or the catalytic and regulatory mechanisms of target enzymes. Regard to pre-mRNA processing, the role of splice junction sites has been widely studied in humans, animal and plant kingdom. However, the correct pre-mRNA splicing not only requires the presence of splicing sites, but also additional intronic and exonic regulatory sequences, involved in the recognition of nearby splice sites [29].

The role of some *cis*-regulatory elements, such as exonic splicing enhancers (ESE) and exonic splicing silencers (ESS), has been investigated in humans, while it remains still poorly understood in plant. In humans it was observed that the mutations located in ESS and ESE elements may modify consensus 5'- or 3'-splice sites, thereby causing aberrant

messengers [30, 31]. Although the alternative splicing in plant is underexplored, compared to the human genome, it has been suggested that genome-wide computational analysis can contribute to provide new knowledge [32].

Here, the production and characterization of high amylose durum wheat has been described, obtained by pyramiding SBEIIa mutations, previously identified [26]. The molecular characterization of a missense mutation responsible for an incorrect processing of *SBEIIa* pre-mRNA is also given.

2. Materials and Methods

2.1 Plant material

The durum wheat cv. Svevo and three *SBEIIa* mutants (*SBEIIa-A*$^-$, *SBEIIa-B*$^-$ and the mutant selection G364E) previously identified using a TILLING [26] were vernalized at 4°C for three weeks. The plants were grown at 20/24°C with a 16 h light period and light intensity of 300 μE m^{-2} s^{-1}. M_5 generation progeny of G364E and F_3 progeny of the cross *SBEIIa-A*$^-$ x *SBEIIa-B*$^-$ were grown in the field. The G364E mutant harbors a missense mutation responsible for a residue alteration in the α-amylase catalytic domain.

2.2. High resolution melting (HRM) genotyping

F_2 progeny bred from the cross *SBEIIa-A*$^-$ x *SBEIIa-B*$^-$ which lacked fully functional *SBEIIa* alleles at both loci were identified using an HRM-based assay. A nested PCR strategy was used, in which the first reaction generated the allele-specific amplicons, as described in Botticella *et al*. [13]. The second reaction, made up to a volume of 10 μL, included as template a 1 μL aliquot of a 1:60 dilution of the first reaction, 5 μL GoTaq® Hot Start Colorless Master Mix (Promega, Madison, USA), 1 μL LCGreen Plus (Idaho Technology Inc., Salt Lake City, USA) and 1.5 μL of each primer to give a final primer concentration of 0.5 μM. The sequence of all of the nested PCR primers used is given in Supplementary Table 1. The PCR program comprised a 95°C/5 min initial denaturation, followed by 39 cycles of 95°C/30 s, 60°C/20 s, 72°C/20 s. At the end of the final extension step, the reaction was held at 95°C for 30 s, then at 25°C for 60 s. The second PCR was run in 96 well Frame-Star plates (4titude Ltd, Surrey, UK) and the "Amplicon genotyping" routine included with the LightScanner instrument (Idaho Technology Inc.) was used to analyze the melting curves.

2.3. Reverse transcription PCR (RT-PCR)

Internal transcripts from the G364E mutant copies of *SBEIIa-A* and *SBEIIa-B* were assayed in 50 uL RT-PCRs containing 25 µL Hot GoTaq®Green Master Mix (Promega), 2 µL cDNA and 0.5 µM of each of the G364E-F and -R primers (Supplementary Table 1). The reactions were subjected to an initial denaturation (95°C/15 min), followed by 39 cycles of 95°C/30 s, 60°C/30 s, 72°C/ 60 s, and a final extension of 72°C/5 min. The amplicons were electrophoresed through a 2% agarose/TBE gel and individual fragments were recovered using a NucleoSpin® Gel and a PCR Clean-up kit (Macherey-Nagel, Düren, Germany). DNA sequencing was performed by Eurofins Genomics (Ebersberg, Germany).

2.4. Quantitative real time (qRT-PCR)

Total RNA was extracted from snap-frozen immature (21 days post anthesis [DPA]) grains of greenhouse-grown plants, following Laudencia-Chingcuanco *et al*. [33], as modified by Sestili *et al*. [12]. The RNA formed the template for the synthesis of cDNA, based on a QuantiTect Reverve Transcription kit (Qiagen, Hilden, Germany), following the manufacturer's instructions. Subsequent 20 µL qRT-PCRs were performed using an iCycler iQ instrument (Bio-Rad Laboratories, Hercules, CA, USA); each reaction contained 10 µL iQ™ SYBR Green Supermix 2X (Bio-Rad Laboratories), 1 µL cDNA and 0.5 µM of each primer. The primer pairs used to assay the transcription of *SSI*, *SSII*, *SSIII*, *SBEI*, *SBEIIb*, *ISA1*, *LD* and *GBSSI* have been reported elsewhere [12]. The *SBEIIa-A* and *SBEIIa-B* primer pair sequences are given in Supplementary Table 1. *Ta2526* was used as the reference sequence, as recommended by Nemeth *et al*. [34]. The $2^{-\Delta\Delta CT}$ method was used to estimate relative levels of transcript abundance [35]. Each qRT-PCR data point represented the mean of three technical replicates for each biological sample. Mean relative transcript abundances were compared using the Student's *t* test.

2.5. Determination of amylose (AC), total starch (TS) and RS content

The starch formed by field-grown F_3 grains from the cross *SBEIIa-A*$^-$ x *SBEIIa-B*$^-$ was characterized from two independent samples per genotype, based on at least six technical replicates per sample. For the double *SBEIIa* mutant, the analyses were

performed on two sister lines. AC was determined from starch isolated following Zhao and Sharp [36], using an iodometric assay developed by Chrastil [37]. A standard curve was prepared using a mixture of potato amylose (Fluka, Neu-Ulm, Germany) and amylopectin isolated from waxy durum wheat. TS and RS content was measured from wholemeal flour using kits provided by Megazyme Pty Ltd. (Wicklow, Ireland). In critical lines, RS was additionally estimated from purified starch. Statistical analysis was performed using Tukey's test.

2.6. Starch granule morphology

Starch was extracted as described above, critical-point dried in a Balzer's apparatus equipped with a liquid CO_2 inlet and metal-shadowed in a gold sputtering unit equipped with an argon inlet. The preparations were examined using a Cambridge Stereoscan 240 scanning electron microscope.

2.7. Isolation and sequencing of the SBEIIa-B *allele*

Genomic DNA was extracted from cv. Svevo and the G364E mutant using a NucleoSpin® Plant II kit (Macherey-Nagel). The entire coding region, split into 22 exons, was amplified using five primer pairs (Supplementary Table 2) in 50 µL reactions comprising 100 ng genomic DNA, 1× GoTaq® Hot Start Colorless Master Mix (Promega) and 0.5 µM of each primer. The amplification regime was as described above for the RT-PCR, except that the extension time was extended to 1.5-3 min. The resulting amplicons were sequenced by Eurofins Genomics (Ebersberg, Germany).

3. Results

3.1. Stacking the SBEIIa null *mutations in cv. Svevo*

In order to combine the two *SBEIIa* null alleles into a single genotype, the F_2 generation of the cross *SBEIIa-A*$^-$ x *SBEIIa-B*$^-$ was assayed using HRM genotyping. The melting curves produced by the amplicons from the double mutant were distinct (Fig. 1). Of the 176 F_2 plants screened, 11 were of genotype *SBEIIa-A*$^-$ */ B*$^-$, 14 carried the wild type alleles at each *SBEIIa* homeologue and the remainder were heterozygous at one or both of the homeologues. The segregation pattern was consistent with digenic inheritance (χ^2=8.02, p>0.05).

3.2. Transcription of starch synthesis genes

A qRT-PCR analysis showed that the transcription of *SBEIIa-A* was reduced by >80% in the double SBEIIa mutant compared to the level in the control sib line in which both *SBEIIa* homeologues were functional. Similarly, the abundance of *SBEIIa-B* transcript was greatly diminished (by about 60%) (Fig. 2). The transcriptional behaviour of the major starch synthesis genes in the double mutant was also characterized. In both independent plants, *SSI*, *SSII*, *SIII* and *GBSSI* were markedly up-regulated compared to the control sib line (Fig. 2). *SBEIIb* was up-regulated by 4-6.5 fold in the double mutant, while the abundance of *SBEI* transcript was about 1.7 fold greater than in the control plants. The genes encoding the debranching enzymes ISA1 and LD were up-regulated in one of the double mutant plants, but not in the other.

3.3. Starch composition of the double mutant

The AC, TS and RS contents and the hundred grain weight (HGW) (Table 1) were determined for the grain of both the single and double *SBEIIa* mutants, and also of the G364E mutant. The amylose content of the grain formed by the *SBEIIa-A*$^{-}$ / *SBEIIa-B*$^{-}$ double mutant ranged from 51.2-52.7%, representing about twice the proportion present in the grain of the control sib line (26.9%). The amylose content in the grain of the single *SBEIIa-A*$^{-}$ and *-B*$^{-}$ mutants was similar to the wild type level (data not shown). The grain set by the G364E mutant contained an unexpectedly high proportion of amylose (54.4%). Both the TS content and the HGW of the double mutant grain were significantly lower (respectively by 12-23% and 9-12%) than those of the control sib line grain. A major increase in the RS content (by up to eight fold) characterized the grain starch formed by the double mutant.

3.4. Starch granule morphology

Scanning electron micrographs demonstrated that the morphology of the A- and B-type starch granules was greatly altered in both the double mutant and the G364E mutant. The granules appeared deformed and deflated, and were less regular and smaller than those in the grain starch of cv. Svevo and the control sib line (Fig. 3). The granules present in the grain starch of the two single mutants were indistinguishable from those formed by cv. Svevo.

3.5. The SBEIIa-B *allele harbored by the G364E mutant*

The G364E *SBEIIa-B* allele was isolated using a primer-walking strategy, as previously reported by Botticella *et al*. [38] (Supplementary Fig. 1). The five amplicons spanning the entire 22 exon coding region were sequenced, and the entire sequence was then compared to that of the allele harbored by cv. Svevo. A single nucleotide G to A polymorphism distinguished the two sequences; its position was adjacent to the splice site at the 3' end of exon 10. A sequence alignment showed that both the alleles (*SBEII-A* and *-B*) shared the same polymorphism at this site (Fig. 4). Since mutations lying close to splice sites are known to affect mRNA processing [39], the RESCUE-ESE web server (http://genes.mit.edu/burgelab/rescue-ese/; [40]) was used to identify local exonic splicing enhancers (ESEs), and the FAS-ESS web server (http://genes.mit.edu/fas-ess/; [41]) to identify any exonic splicing silencers (ESSs). The former predicted no change in pre-mRNA processing, but the latter suggested that the sequence variant in G364E induced the loss of an ESS site (TTTGGG) between the end of exon 10 and the splice junction site (Fig. 5). To experimentally validate this prediction, RT-PCR was applied to mRNA extracted from 21 DPA grains, based on a primer pair spanning exons 10-11. Three amplicons were generated from both of the *SBEIIa* homoeloci: one was of the expected size (154 bp) and was also amplified from a cv. Svevo template; but there were two additional ones (342 bp and 191 bp) for *SBEIIa-A* and two further ones (339 bp and 188 bp) for *SBEIIa-B* which were specific to the G364E mutant (Fig. 6a). Sequencing of the longer non-wild type amplicons confirmed that they retained the sequence of intron 10. Sequencing of the shorter non-wild type amplicons suggested the presence of an alternative splicing site (Fig. 6b), located 36 nt (*SBEIIa-A*) and 33 nt (*SBEIIa-B*) downstream of the wild type one (Figs 4 and 6b). The aberrant transcripts did not encode a functional SBEIIa enzyme, since they included a premature stop codon; however, since a portion of the transcript was normally processed, the likelihood is that some transcript was translated into a functional SBEIIa polypeptide. To evaluate whether the full length G364E transcript caused any meaningful change in either the secondary or and tertiary structure of the product, the predicted polypeptide sequences were analysed by the I-TASSER web-free server (http://zhanglab.ccmb.med.umich.edu/I-TASSER/) and the SWISS-MODEL web-free server (http://swissmodel.expasy.org/interactive); no differences between the wild type and the mutant sequences were identified.

4. Discussion

A breeding strategy has permitted to pyramid SBEIIa knock out mutations, previously identified in a durum wheat TILLING platform [26]. The suppression of the *SBEIIa* has produced pleiotropic effects on other starch biosynthetic genes. Just as it has been observed here for the durum wheat double mutant, bread wheat lines harbouring compromised *SBEIIa* genes also show a reduced level of *SBEIIa* transcripts [13, 15]. The origin of this reduction is thought to involve the nonsense mediated mRNA decay mechanism, which imposes a measure of control over mRNA quality, and inhibits the formation of non-functional (truncated) translation products [42, 43]. The knock-down of *SBEIIa* transcription resulted in an up-regulation of the genes encoding starch branching enzymes I and IIb, the starch debranching enzymes ISA1 and LD, and the full set of starch synthases, possibly reflecting the activation of a compensatory mechanism, though the increase in transcript amount may not lead to changes in enzyme activity. The outcomes of the present transcriptional analysis differ somewhat from those described in bread wheat *SBEIIa* mutants by Slade *et al*. [15], where there was no difference between the *SSI*, *GBSSI* or *SBEIIb* transcript abundance in the *SBEIIa* triple mutant and the wild type. However, the effect of knocking out *SBEIIa* in barley, as in durum wheat (present results and Sestili *et al*. [12]), was to up-regulate *SSI*, *SSIIa* and *GBSSIb* [44]. More widely, altering the transcription of individual starch synthesis genes often generates a pleiotropic effect on other starch synthesis related genes not only in wheat, but also in rice, barley and maize [45-49]. The effect of knocking down *SBEIIa* expression on grain amylose content agrees well with the observations of Slade *et al*. [15], who noted a similar increase (94%) in the amylose content of durum wheat null *SBEIIa* lines, but differs somewhat from the results of Hazard *et al*. [14], who reported a more modest increase (22%). The latter authors combined a *SBEIIa-A* knock-out mutant with an *SBEIIa-B* allele in which the splicing acceptor site at the end of intron 8 had been lost; however the extent to which the latter mutation disrupted the function of SBEIIa-B was not ascertained. More recently, Hazard *et al*. [28] have combined the same two mutations with two *SBEIIb* mutations to obtaining a *SBEIIa/SBEIIb* knockout line, in which both the amylose and the RS contents resembled their levels in the present double mutant. At the hexaploid level, the proportion of amylose present in the grain of both the *SBEIIa* triple mutant and of *SBEIIa* RNAi

knockouts ranged from 70% to 85% [9, 15, 50]. Why the effect of compromising *SBEIIa* in bread wheat is more marked than in durum wheat remains unclear. A possible hypothesis is that this difference is due to the genetic background as demonstrated by Howard et al. [51] that introgressed different mutations into a barley elite cultivar and have shown that some starch characteristics are strongly influenced by the genetic background.

Both the TS content and the TGW, traits which are associated with high grain amylose content [12, 15, 16, 52], of the double mutant were lower than in the sib control line. Since RS is positively correlated with amylose content, it was markedly enhanced in the double mutant, just as observed by Slade *et al*. [15]. With respect to their morphology, the double mutant starch granules were distorted, in ways similar to those in wheats lacking SBEIIa activity or the Sgp-1 protein [8, 9, 12, 15, 16].

Surprisingly the biochemical characterization (AC, TS, RS) and scanning electron microscopy analysis of starch granules showed that the mutant line G364E had a phenotype similar to the complete null SBEIIa genotypes. Further investigation confirmed the presence of the same mutation on both SBEIIa alleles. As we estimated a mutation rate of 1/47 kbs in the TILLING population [26], the probability, that this event occurs in the same plant, is extremely rare. In the literature no report has been found describing a similar case in mutagenised plant species. Another possibility is that one of the two mutations has been generated through crossing over or gene conversion.

ESEs and ESSs represent regulatory elements in both the plant and animal kingdoms [32, 39, 40, 53-56]. The loss of the ESS motif from exon 10 in both *SBEIIa* copies carried by the G364E mutant was confirmed *in vitro* to generate mRNA variants. None of the aberrant transcripts were translated into a functional SBEIIa enzyme, as they all generated truncated translation products. However a portion of the transcript was correctly processed. The hypothesis is that the G364E mutation, which is located in the enzyme's catalytic domain, contributed to affect the functionality of SBEIIa enzymes in the mutant G364E.

5. Conclusions

Combining the two knock down *SBEIIa* mutant alleles by intercrossing the single mutants has facilitated the breeding of durum wheat with a high relative content of amylose and a high level of RS. These materials could find application in elaborating

food products with superior technological and/or nutritional characteristics. The characterization of the G364E mutant has highlighted some interesting structural features of *SBEIIa*. In particular, the ESS motif at the end of exon 10 is clearly essential for the correct processing of pre-mRNA. In addition, the glycine residue at position 364 could have a role for the functionality of SBEIIa.

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Figure legends

Fig. 1. HRM genotyping of a selection of F_2 progeny bred from the cross *SBEIIa-A*$^-$ x *SBEIIa-B*$^-$. Wild type homozygotes, mutant homozygotes and heterozygotes at *SBEIIa-A* are shown in grey, blue and red, respectively.

Fig. 2. The transcription in 21 DPA grains of *SSI*, *SSII*, *SSIII*, *SBEI*, *SBEIIa-A*, *SBEIIa-B*, *SBEIIb*, *GBSSI*, *ISA1* and *LD* as assayed by qRT-PCR. Data are shown as fold differences in transcript abundance between the control sib line and the double mutant. Dotted line indicates the relative transcription value of the control. Data relative to the first and second biological samples are indicated with light and dark grey histograms, respectively. Standard error bars are indicated. Genes significantly ($P<0.05$) up- or down-regulated are marked by an asterisk (*); ns: no significant difference in transcript abundance.

Fig. 3. Scanning electron micrographs showing the starch granules isolated from mature grains of cv. Svevo, the control sib line null segregant (NS), the G364E mutant, the single *SBEIIa* mutants (*SBEIIa-A*$^-$ and *SBEIIa-B*$^-$) and the double mutant (*SBEIIa-A*$^-$*B*$^-$) genotypes.

Fig. 4. Nucleotide alignment between the *SBEIIa-A* and *-B* sequences of the line G364E and cv. Svevo. The asterisks indicate the two point mutations identified in the SBEIIa mutant line G364E. Red boxes contain SNPs which discriminate between the *SBEIIa-A* and *-B* alleles. Black and green boxes highlight the normal and alternative splice sites.

Fig. 5. The *SBEIIa* region between exons 10 and 11, as analysed by FAS-ESS software. The asterisks indicate the sequence polymorphisms present in the G364E mutant sequences. The black box indicates an ESS motif lost in the mutant line.

Fig. 6. (A) The amplicons generated by RT-PCR of 21 DPA grains from the region spanning *SBEIIa* exons 10 and 11. 1) cv. Svevo, 2) G364E mutant. M: size marker. A schematic representation of (B) pre-mRNA and (C) spliced mRNAs produced by the G364E mutant. Exons represented by grey boxes and introns by horizontal lines. Lines above and below the introns indicate the two alternative splicing sites identified.

Table 1. RS, TS, AC and HGW of the various lines. Different letters indicate significant differences from the performance of the control sib line (P<0.05). Values are given as mean and SE (*n*=2, three technical replicates per each biological sample). In the case of the double mutant, two sister lines were analysed. Due to inadequate seed availability, neither TS nor HGW were determined for the G364E mutant. Asterisks indicate RS values determined from purified starch.

Genotype	Resistant Starch (g/100g)	Total Starch (g/100g)	Amylose %	Hundred grain weight (g)
NS	0.82±0.16[a] (*0.47±0.01)	58.9±1.0[a]	26.9±1.40[a]	4.1±0.1[a]
SBEIIa null-1	6.47±0.10[b]	45.1±0.2[b]	52.7±0.70[b]	3.7±0.1[b]
SBEIIa null-2	6.79±0.33[b] (*7.89±0.08)	51.4±0.2[b]	51.2±1.02[b]	3.6±0.3[b]
SBEIIa G364E	nd	nd	54.4±0.44[b]	nd

Highlights

Generation and identification of single point mutations in *SBEIIa* genes of durum wheat

Pyramiding of the two non-sense mutations results in a high amylose phenotype

Characterization of a line with the same missense mutation in both SbeIIa gene homeoalleles

Figure 1

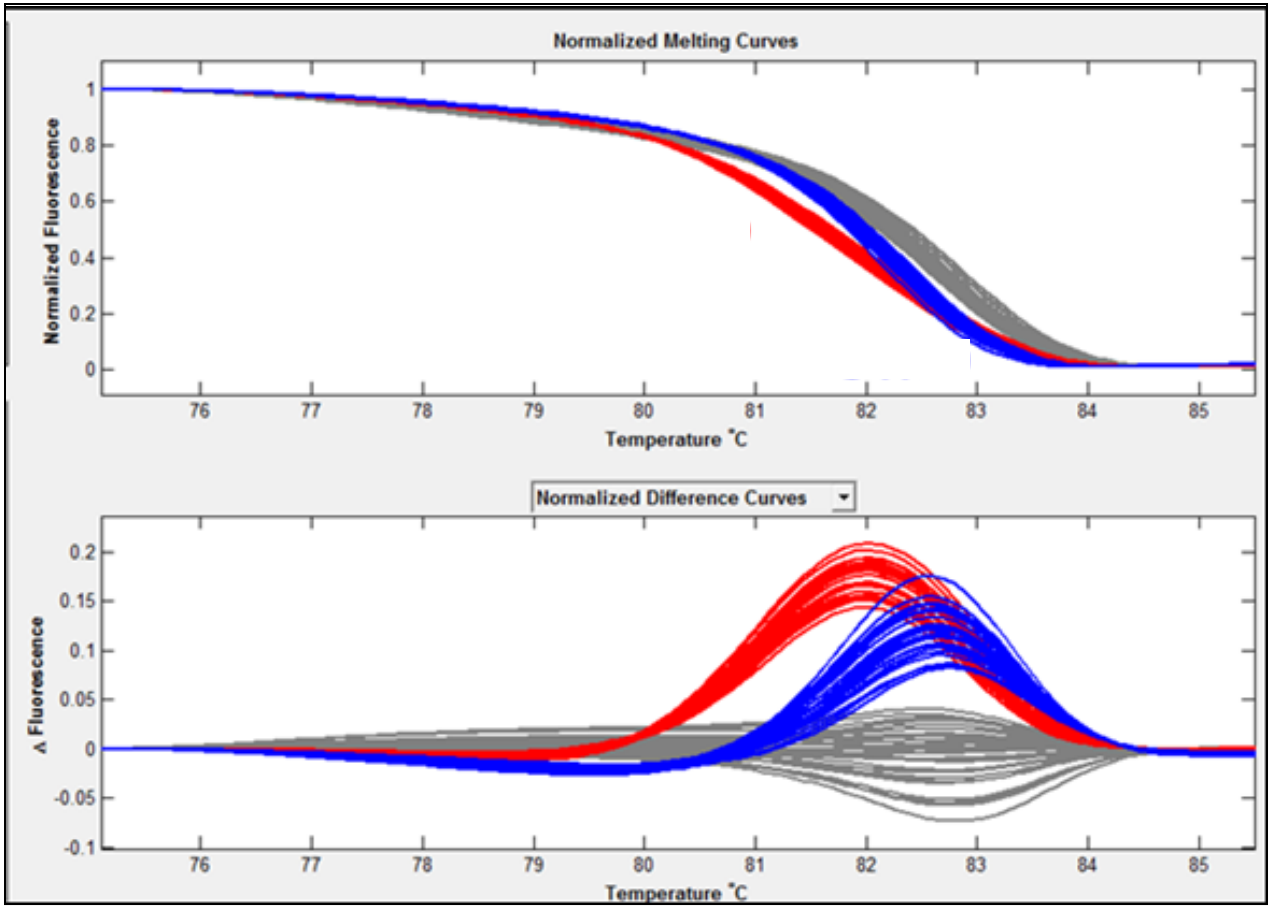


Figure 1

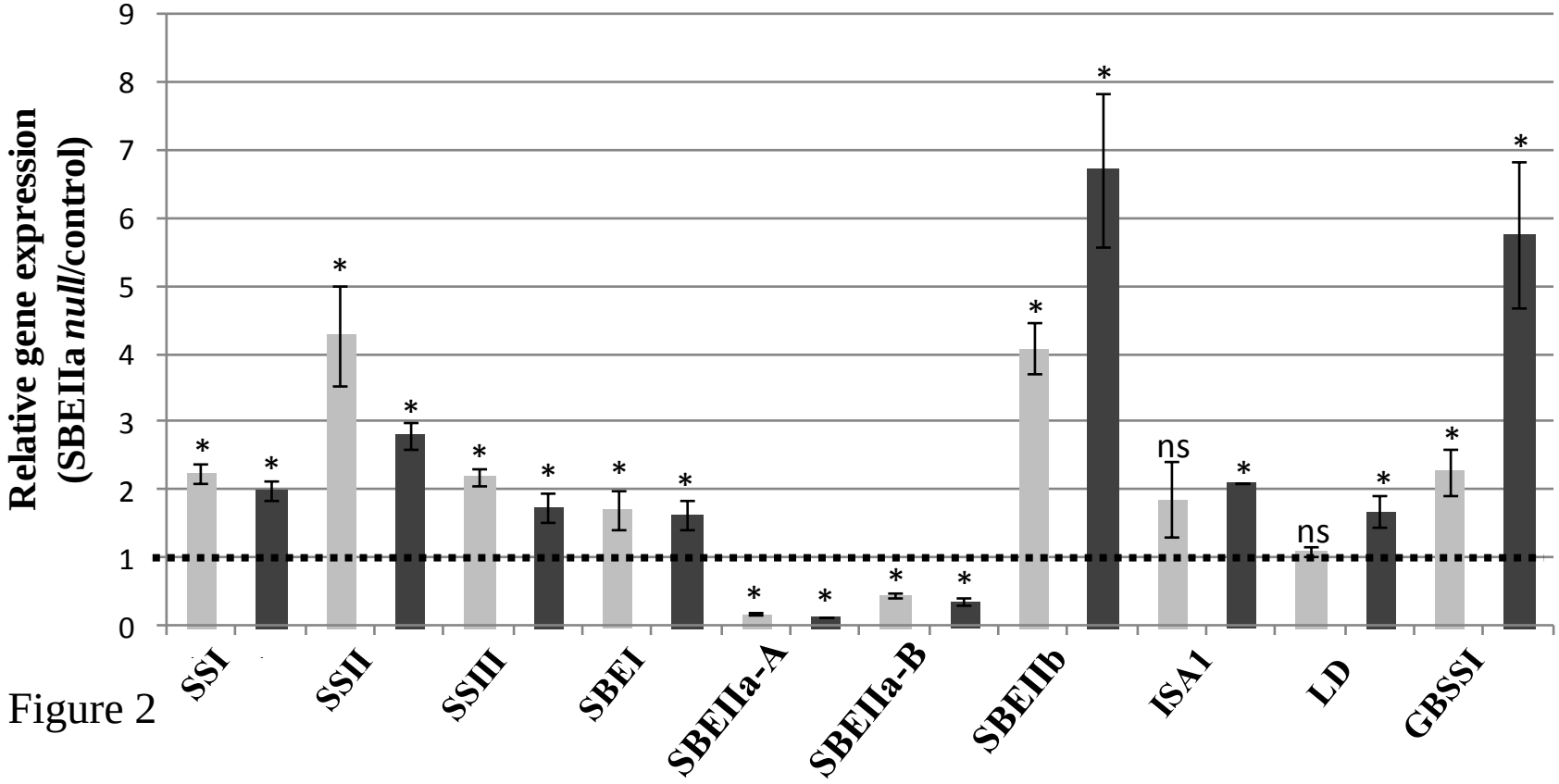

Relative gene expression
(SBEIIa *null*/control)
9
8
7
6
5
4
3
2
1
0
*
ns
SSI
SSII
SSIII
SBEI
SBEIIa-A
SBEIIa-B
SBEIIb
ISA1
LD
GBSSI

Figure 2

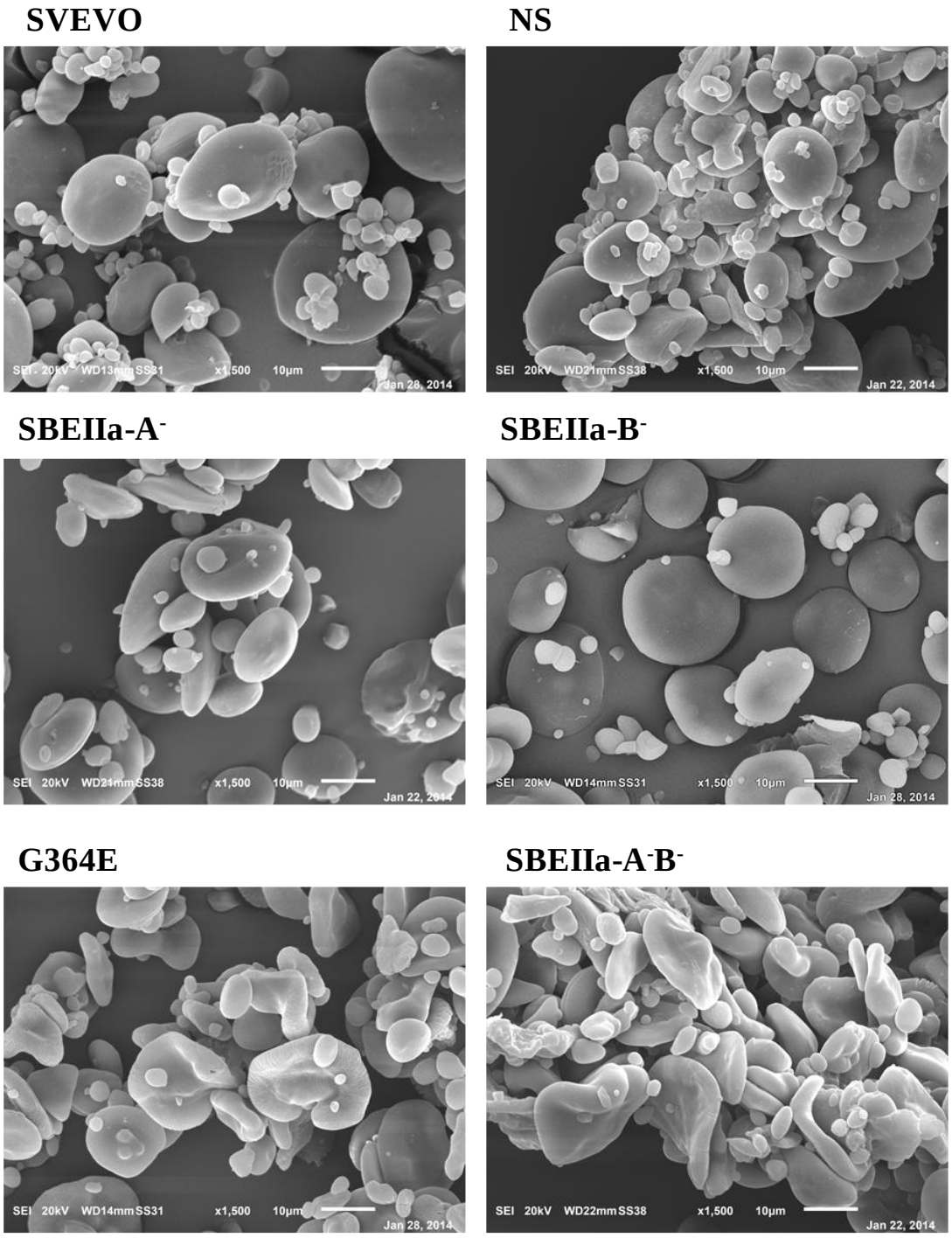

SVEVO
NS
SEI 20kV WD13mm SS31 x1,500 10µm Jan 28, 2014
SEI 20kV WD21mm SS38 x1,500 10µm Jan 22, 2014
SBEIIa-A-
SBEIIa-B-
SEI 20kV WD21mm SS38 x1,500 10µm Jan 22, 2014
SEI 20kV WD14mm SS31 x1,500 10µm Jan 28, 2014
G364E
SBEIIa-A-B-
SEI 20kV WD14mm SS31 x1,500 10µm Jan 28, 2014
SEI 20kV WD22mm SS38 x1,500 10µm Jan 22, 2014

Figure 3

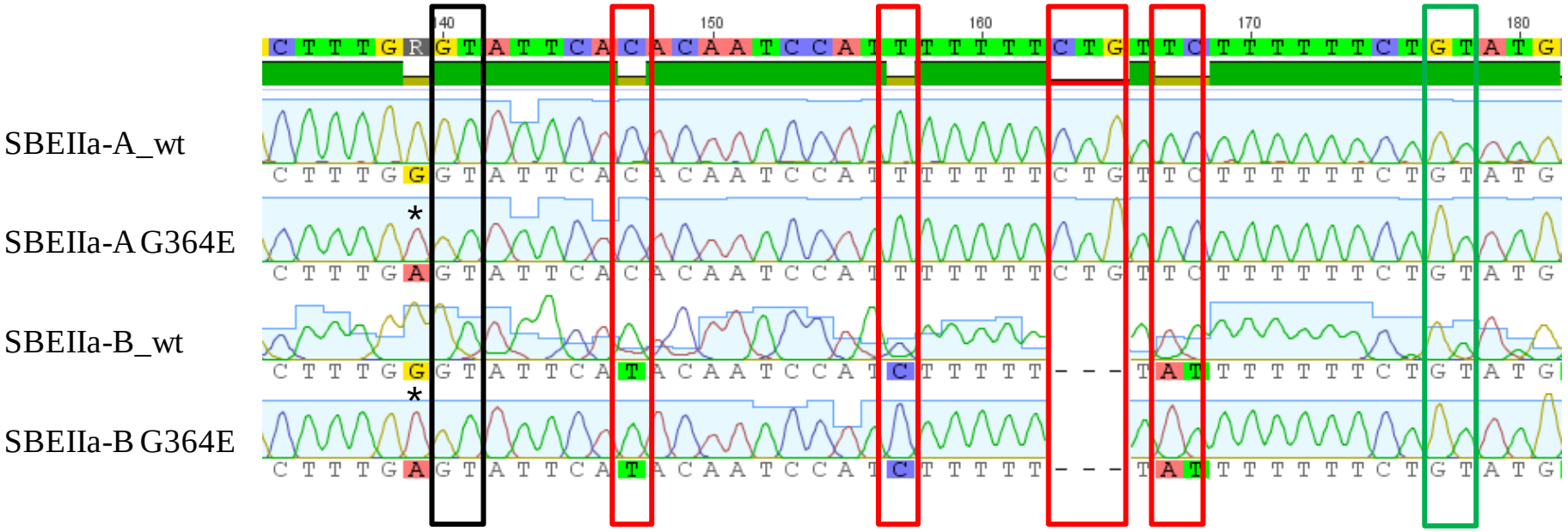
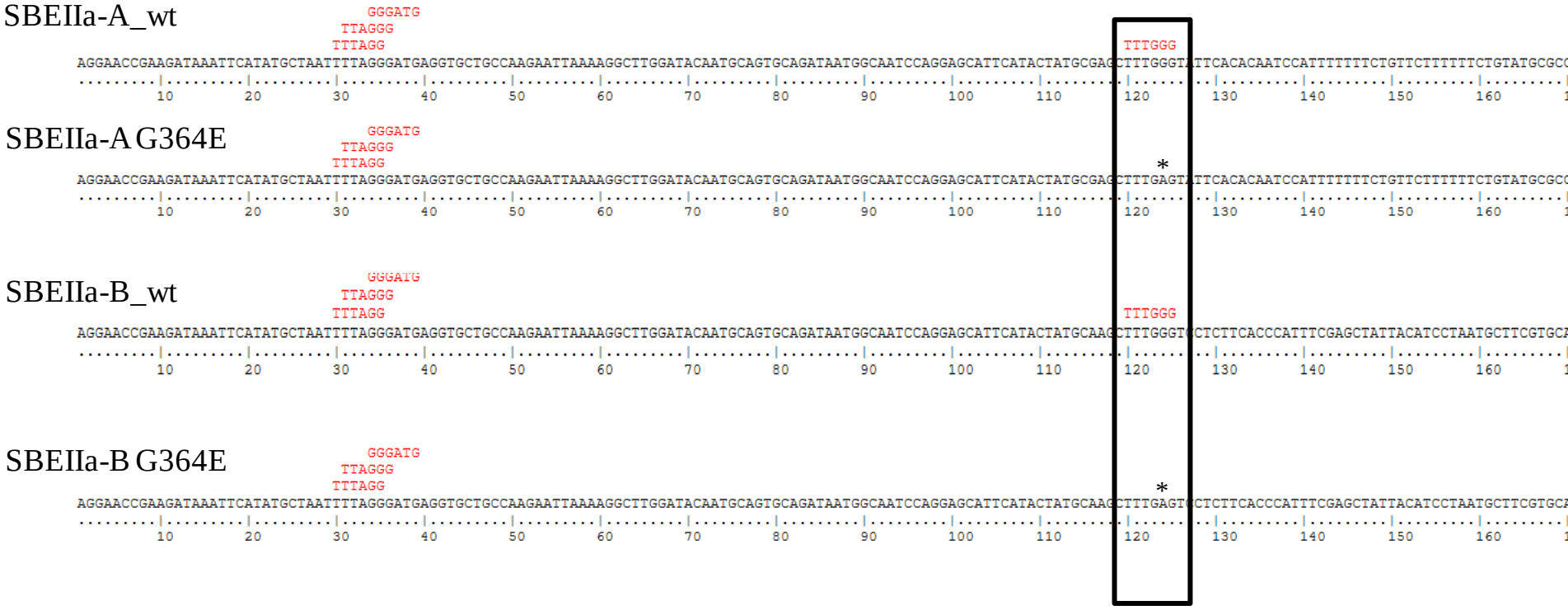

SBEIIa-A_wt
SBEIIa-A G364E
SBEIIa-B_wt
SBEIIa-B G364E
140
150
160
170
180

Figure 4


SBEIIa-A_wt
GGGATG
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SBEIIa-A G364E
GGGATG
TTAGGG
TTTAGG
*
SBEIIa-B_wt
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SBEIIa-B G364E
GGGATG
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TTTAGG
*

Figure 5

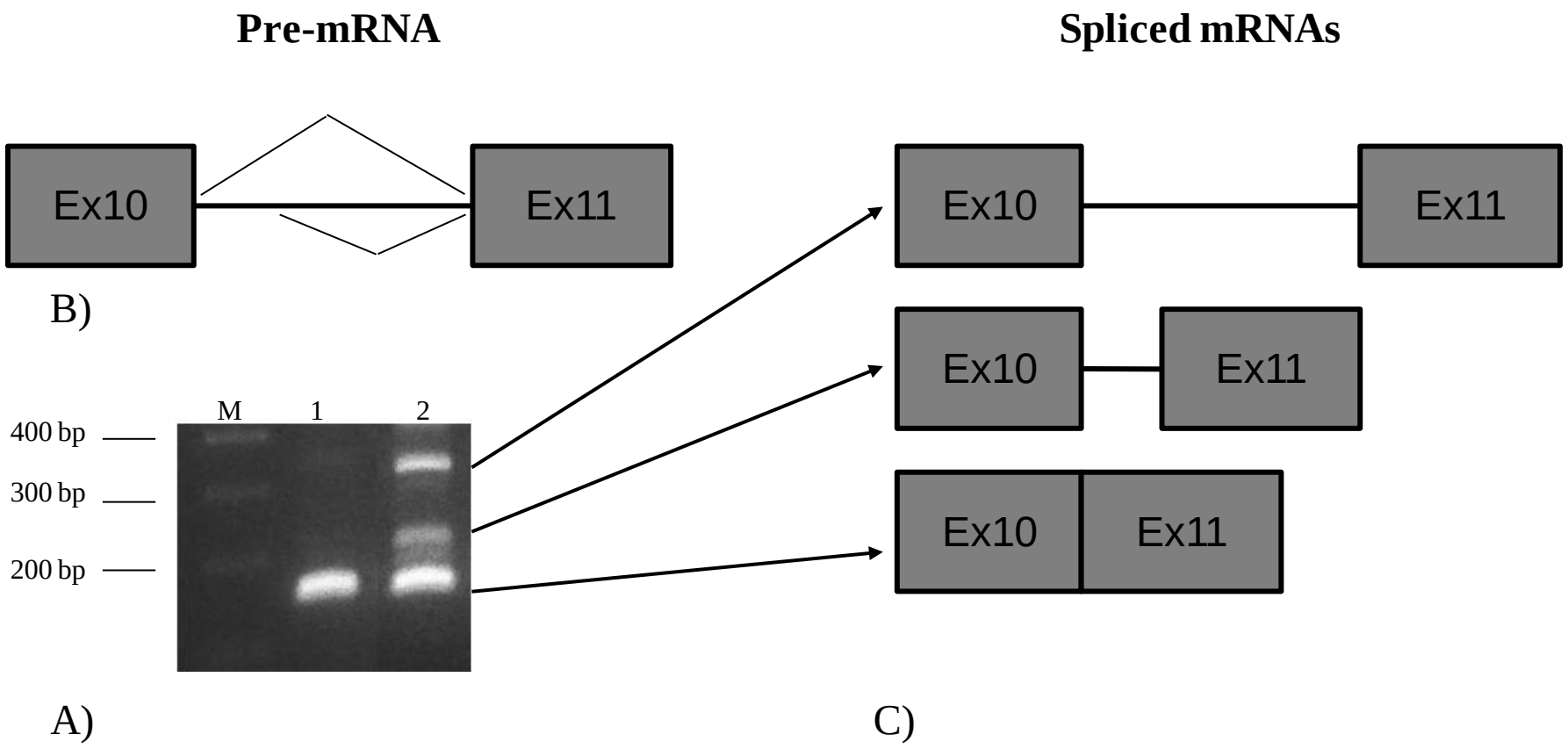

Figure 6