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1 **The cell wall active mannuronan C5-epimerases in the**
2 **model brown alga *Ectocarpus*: from gene context to**
3 **recombinant protein**

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19 List of Supplementary Data: Table S1 to S2 and Fig.S1 to S8

1 Abstract (182 words)

2 Mannuronan C5-epimerases (ManC5-Es) catalyse in brown algae the remodelling of alginate,
3 a major cell wall component which is involved in many biological functions in these
4 organisms. ManC5-Es are present as large multigenic families in brown algae, likely
5 indicating functional specificities and specialisations. ManC5-Es control the distribution
6 pattern of (1-4) linked β -D-mannuronic acid (M) and α -L-guluronic acid (G) residues in
7 alginates, giving rise to widely different polysaccharide compositions and sequences,
8 depending on tissue, season, age or algal species. As such they are also a source of powerful
9 new tools for the biotechnological and enzymatic processing of alginates, to match the
10 growing interest for food hydrocolloids and in biomedical and nanotechnological applications.
11 We report here the first heterologous production of a ManC5-E of brown algal origin that is
12 successfully refolded in an active form. The activity was measured by $^1\text{H-NMR}$ and by an
13 indirect enzymatic assay using a known bacterial alginate lyase. The transcript expression as a
14 function of the developmental program of the brown alga *Ectocarpus*, together with the
15 bioinformatic analyses of the corresponding gene context of this multigenic family, are also
16 presented.

17

18

19

1 **Introduction**

2

3 Alginate is a major cell wall constituent of brown algae (Phaeophyceae) also synthesized by
4 some bacteria of the *Azotobacter* and *Pseudomonas* genera. This polysaccharide is of
5 important commercial value with an annual worldwide production of about 45,000 tons,
6 extracted from various types of brown seaweeds including species mostly from the orders of
7 Laminariales and Fucales (Smidsrod and Draget 1996). In many different applications,
8 alginates act as stabilizing, thickening, and/or gelling agents. The main uses are food
9 additives. Alginates are increasingly used in cosmetics, health care and pharmaceuticals (Zhao
10 et al. 2012) and lately received a renewed interest for their use in nanotechnology (Kovalenko
11 et al. 2011) and in fuel and ethanol productions (Enquist-Newman et al. 2014, Wargacki et al.
12 2012, Wei et al. 2013).

13 Alginate is a linear copolymer of 1,4-linked uronic acid epimers differing only at C5:
14 the β -D-mannuronic acid (M) and the α -L-guluronic acid (G). These residues are arranged in
15 homopolymeric regions of MM- and GG-blocks, interspaced with random arrangements of
16 both monomers (MG-blocks), each of which have different conformational preferences and,
17 hence, different physico-chemical properties (Donati et al. 2005, Heyraud et al. 1996). While
18 the MM-block regions do not associate in the presence of divalent cations, GG-blocks regions
19 will form 'egg-box' junctions with calcium (Grant et al. 1973), bridging two antiparallel
20 chains and leading to gel formation. The mechanical strength of an alginate gel thus correlates
21 with the proportion of GG-blocks (Figure 1). Alternating structures take up rather disordered
22 conformations, yielding more flexible gels.

23 G-rich alginates with a M/G ratio ≤ 1.0 constitute around 25% of the total alginate
24 market, with a yearly growth of about 10%, mainly in the food market. This type of alginate is
25 also widely used in the pharmaceutical area (Draget and Taylor 2011). As most seaweed

1 species have a M/G ratio of 1.2-1.6 in alginate (Haug et al. 1974, Smidsrod and Draget 1996),
2 brown seaweeds with a naturally low M/G ratio of 0.7-0.5 are in high demand. Alginate with
3 an M/G ratio of 0.4 is exclusively found in stipes of *Laminaria hyperborea* and *Lessonia*
4 *trabeculata* (Smidsrod and Draget 1996). The M/G ratio of alginate from the whole *L.*
5 *hyperborea* plant is 1.0 (Draget et al. 2005) and separation of fronds from stipes is laborious.
6 Thus, novel ways to ensure an accessible and efficient production of alginate with low M/G
7 ratios are desirable. Although chemical epimerisation can be performed on the polysaccharide
8 (Pawar and Edgar 2012), the high temperature required for this process leads to undesirable
9 depolymerisation. At lower temperature actual processes use chemical catalysts that are
10 difficult to remove from polyanionic material with strong cationic complexing properties,
11 such as alginates.

12 The alginate biosynthesis pathway is yet not fully resolved in brown algae but genes
13 homologous to their bacterial counterparts have been identified (Michel et al. 2010). The final
14 steps were proposed to be the production of polymannuronate and the subsequent
15 epimerization on the polymer of the M residues into G residues; the latter being catalysed by
16 putative mannuronan C5-epimerases (ManC5-Es) resembling bacterial ManC5-Es (Nyvall et
17 al. 2003). The biochemical characterisation of native and hybrid ManC5-E of bacterial
18 sources has shown their abilities to generate distinct alginate structures (Wolfram et al. 2014,
19 Bjerkan et al. 2004, Hartmann et al. 2002). The presence of a large number of ManC5-E
20 genes in brown algae also suggests that their gene products might yield polymers with
21 different patterns of G and M distributions. Given that alginates in brown algae affect the
22 texture of the cell wall in a similar manner as do pectins in the cell walls of land plants, this
23 multigene situation is reminiscent of pectin methyl-esterases (PMEs) (Micheli 2001). By
24 analogy to PMEs, which can yield partially methylesterified pectins with either random or
25 blockwise distribution of the methyl groups, the different isoforms of ManC5-E might yield

1 alginates with different patterns of GG-block distribution. Moreover, the diversity of enzymes
2 present in brown algae to remodel the algal cell wall polysaccharide, is much larger than that
3 found in bacterial alginate synthesizing systems (Bjerkkan et al. 2004). It can thus be assumed
4 that these algal enzymes could be used to produce alternative “tailor-made alginates” with
5 novel physico-chemical properties

6 Heterologous expression of algal genes is notoriously difficult and has presented a
7 bottleneck in projects aiming at characterising enzymes of macroalgal origins (Groisillier et
8 al. 2010, Kurland and Gallant 1996). Recent access to the first genome sequence of a brown
9 alga (Cock et al. 2010) allowed the identification of all the genes shaping the ManC5-E
10 family in *Ectocarpus*. By providing multiple targets, it opened a new door toward the
11 expression of recombinant ManC5-Es. We took advantage of a medium throughput strategy
12 we recently developed for protein expression (Groisillier et al. 2010), to heterologously
13 express the ManC5-Es from *Ectocarpus*. Our work has generated the first recombinant and
14 active ManC5-E from a brown alga. An additional comprehensive analysis of the gene
15 context and transcript expression during the life cycle of *Ectocarpus* has provided new
16 insights into the possible roles of ManC5-E genes in the brown algae.

1 **Results**

2

3 ***Ectocarpus* contains a multigenic family of putative ManC5-Es**

4 The functional annotation of the genome of *Ectocarpus* previously indicated the occurrence of
5 28 genes encoding putative mannuronan C5-epimerases (ManC5-Es) (Michel et al. 2010). A
6 more detailed analysis led to the identification of additional genes that shape a final
7 multigenic family of 31 members that accounts for 0.19% of the total predicted gene number
8 in *Ectocarpus*. The gene names and the corresponding acronyms (which use a 5-characters
9 code) are listed in Supplementary Table S1. For phylogenetic analyses, 10 genes were
10 discarded due to doubtful predicted gene structure. The phylogenetic tree indicated that at
11 least four groups of ManC5-Es can be identified in *Ectocarpus* (Figure 2A). Among them one
12 group contains modular proteins that are predicted to contain additional modules appended to
13 the catalytic ManC5-E domain: a schematic diagram showing the protein structure of the eight
14 candidates is shown in Figure 2B and Supplementary Figure S1. Five of the eight encoded
15 proteins are predicted to contain a signal peptide targeting the protein to the cell surface. In
16 addition to the catalytic ManC5-E domain, three main supplementary domains are found: the
17 Wall Sensing Component (WSC), the AGP-protein core domain and a module of unknown
18 function. The WSC domains are known to be involved in cell-wall sensing in yeast. They also
19 shape a large multigenic family in the phylogenetic-distant brown algae where they are
20 expected to play similar functions (Cock et al. 2010, Hervé et al. 2016). Arabinogalactan-
21 proteins (AGPs) are highly glycosylated, hydroxyproline-rich proteins found at the cell
22 surface of plants, but they have also been recently investigated in brown algae (Hervé et al.
23 2016). In both systems they play key roles in developmental processes. The module of
24 unknown function (MUF) was identified manually and up to now we could not detect any
25 significant homology to other proteins or domains deposited in public databases. The MUF

1 sequence is highly conserved (Supplementary Figure S2) and is often found as repetitive
2 motifs in the ManC5-E sequences under concern (i.e. MEP3, MEP1, MEP4) (Figure 2B). The
3 protein motif consists of a sequence of 56 amino acids in average, and displays a particular
4 amino acid composition (cysteine-, glycine- and serine-rich) (Supplementary Figure S2). Such
5 particular amino acid patterns are often unstructured, i.e. linkers, or display propensity to self-
6 assemble and we thus speculate that these domains might play a role in structuring these
7 proteins within the cell wall.

8

9 **The ManC5-E family includes genes that are differentially regulated during the life-**
10 **cycle of *Ectocarpus***

11 Thirty of the 31 predicted ManC5-E gene family members are transcribed during the life
12 cycle of *Ectocarpus* (<http://bioinformatics.psb.ugent.be/orcae/overview/EctsiV2>). The
13 remaining gene that exhibits no expression in any of the conditions tested (MEP30), contains
14 a large insertion of the *Ectocarpus* virus (EsV1-163) which may alter gene expression. The
15 life cycle of *Ectocarpus* involves an alternation between two morphologically distinct
16 generations, the sporophyte and the gametophyte (Cock et al. 2015). Microarray expression
17 data (Coelho et al. 2011) obtained for a subset of the ManC5-E genes revealed an interesting
18 expression pattern during the life cycle generation stages. While the majority of these twelve
19 genes were indistinctively expressed in both generations of the life cycle, four ManC5-E
20 genes (MEP6, MEP26, MEP27, MEP28) were preferentially expressed in the gametophyte
21 generation (Figure 3, *t*-test $P < 0.05$), suggesting a role for these genes in the development of
22 the gametophyte. These four genes belong to different ManC5-E phylogenetic clusters.
23 Therefore, within a common cluster of ManC5-E genes, some can be either mostly expressed
24 in the gametophytes, or showing no difference in their expression pattern. We conclude that
25 the expression of some of the ManC-Es is differentially regulated during the life cycle of

1 *Ectocarpus* and that the ManC5-Es do not cluster in the phylogenetic tree in relation to their
2 expression patterns.

3

4 **Gene structure analyses**

5 Gene exon-intron structures analyses of the ManC5-E family were conducted. Figure 2C
6 shows the mosaic structure of all the ManC5-E genes included in the tree. The average gene
7 length of ManC5-E genes is larger than the total average gene length in *Ectocarpus* (Table
8 1A). In addition most ManC5-E genes possess more than 9 introns, with the average intron
9 number being nearly twice that of the other *Ectocarpus* genes. The most common system for
10 getting recombinant proteins used bacterial strains derived from *Escherichia coli*. The
11 ManC5-E genes from *Ectocarpus* were examined regarding the codon usage preference: three
12 of the 5 rare codons for *E. coli* show critical relative adaptiveness values (Table 1B). All these
13 characteristics are likely to render heterologous expression of the ManC5-E genes difficult
14 and we payed special attention during the process (described below) to overcome this
15 problem.

16

17 **Expression, purification and refolding of the mannuronan C5-epimerase MEP13**

18 Although different strategies were used (e.g. *E. coli* strains, mammalian cells, in vitro assays),
19 our previous attempts during the past fifteen years to express two putative ManC5-Es from
20 *Laminaria digitata* (Nyvall et al. 2003), were proved unsuccessful. The genome sequence of
21 *Ectocarpus* offers the opportunity to have access to a higher number of genes, and potentially
22 proteins. In order to increase our chances of getting soluble expression of active and
23 recombinant ManC5-Es, a medium throughput strategy was applied (Groisillier et al. 2010).
24 The 31 putative ManC5-E genes were analysed and the corresponding encoded catalytic
25 domains tentatively expressed. The medium throughput strategy rapidly showed that

1 obtaining soluble heterologous protein expression of this eukaryotic protein family in
2 bacterial expression systems was challenging, if not impossible. Attempts to express some
3 ManC5-E genes in soluble form in other organisms than *E. coli* (i.e. the bacterium
4 *Lactococcus lactis*, the yeasts *Pichia pastoris* and *Hansenula polymorpha*, and insect cells)
5 were not successful. The targeted catalytic module of MEP13 (MEP13-C5) was insolubly but
6 highly expressed in *E. coli* (Supplementary Figure S3A), and our efforts in getting an active
7 recombinant ManC5-E were subsequently focused on this specific target. In order to
8 overcome a codon usage preference in *E. coli* strains, we used a codon-optimized sequence of
9 *MEP13-C5*, the latter one being designated *MEP13-C5_{syn}* (Table 1B and Supplementary
10 Figure S4). None of the different media or strains used improved the soluble expression of
11 MEP13-C5, and a high level of insoluble protein was obtained in all cases (Supplementary
12 Figure S3A,C). Nevertheless, usage of different complex media such as TB resulted in a
13 higher cell density and consequently in a higher amount of inclusion bodies. In order to
14 increase our chances of getting recombinant proteins, we included two additional *E. coli*
15 strains developed for the expression of toxic protein or rare codon containing genes. Usage of
16 *E. coli* Rosetta-gami2(DE3)pLysS produced larger and more significant inclusion bodies than
17 *E. coli* BL21CodonPlus(DE3)RIPL. This discrepancy might be due to the different growth
18 rates of these strains. Rosetta-gami2(DE3)pLysS grows more slowly than
19 BL21CodonPlus(DE3)RIPL and incubation overnight seems to be more suitable to obtain the
20 targeted protein expression with this strain.

21 The heterologous expression of MEP13-C5 in *E. coli* Rosetta-gami2(DE3)pLysS
22 yielded about 15 mg/L of insoluble enzyme. The His-tag containing protein was then
23 successfully refolded using an on-column refolding procedure. The analysis of the SDS-
24 PAGE gels and corresponding western-blot (Supplementary Figure S3A,B) showed that after
25 eluting the refolded protein, the samples were essentially pure, also indicating that only large

1 aggregates, eluted in the flow-through, or refolded protein were present, yielding a final
2 quantity of 6.5 mg of active MEP13-C5 enzyme. The identity of the protein was confirmed by
3 peptide mass fingerprinting (Supplementary Figure S5). The DLS spectra of the protein
4 solutions showed high monodispersity of the refolded samples, as compared to the unfolded
5 protein samples (Supplementary Figure S6).

6

7 **The refolded MEP13-C5 shows a mannuronan C5-epimerase activity**

8 The activity of the refolded MEP13-C5 was monitored by measuring the change of M/G ratio
9 in two different alginate solutions (having an initial M/G ratio of 1.5 and 1.7, respectively)
10 using ¹H-NMR. In both cases a decrease of the M/G ratio indicated the action of MEP13-C5,
11 epimerizing some mannuronate units into guluronate (Figure 4A). The change of blockiness
12 could be analysed by integrating and measuring the relative surface of the NMR-peaks that
13 appear in the range of 4.4.40 to 5.15 ppm (Figure 4B,C, Supplementary Figure S7), following
14 the method described previously (Grasdalen et al. 1981, Grasdalen 1983, Hartmann et al.
15 2002). The increase of G-block structure (Figure 4B) is observed with both alginates but is
16 only significant for the sample with a M/G-ratio of 1.5. Figure 4C illustrates that for both
17 alginate samples, the repeated MM structures disappear while GG and GGG structures
18 significantly increase. However, these data do not yet allow clear and full identification of
19 which patterns are preferentially recognized and which are produced by the enzyme.

20 An additional activity assay, using a GG-specific alginate lyase (AlyA1; Thomas et al.
21 2013), was set up that allowed indirect evidence of ManC5-E activity. Indeed, under identical
22 conditions, the MEP13-C5 pre-treated alginate samples produced up to 1.5-fold higher
23 abundance of unsaturated sugar units upon incubation with the GG-specific alginate lyase
24 than the initial untreated alginate samples (Figure 5).

1 **Discussion**

2

3 **Functional validation of a putative mannuronan C5-epimerase gene from brown alga**

4 To date, fully biochemically characterized mannuronan C5-epimerases have only been
5 reported for enzymes of bacterial origin (Buchinger et al. 2014, Campa et al. 2004, Hartmann
6 et al. 2002, Rozeboom et al. 2008, Wolfram et al. 2014). Nyvall et al. (2003) were the first to
7 isolate full length cDNAs from a brown alga (*Laminaria digitata*) displaying similarity with
8 bacterial mannuronan C5-epimerase genes. They proposed that these genes encoded
9 functional ManC5-Es involved in the terminal step of alginate biosynthesis in brown algae.
10 Analysis of the genomic sequence of *Ectocarpus* provided candidate genes for the whole
11 alginate biosynthetic pathway, including the putative ManC5-E genes (Michel et al, 2010).
12 Nonetheless, the predicted function of these brown algal genes has never been biochemically
13 proven until now. Our study reports, for the first time, the heterologous production, refolding
14 and purification of an active ManC5-E, namely MEP13-C5, of brown algal origin. At least for
15 the here reported gene, this result thus validates the hypothesis formulated by Nyvall et al.
16 (2003) twelve years ago. To our knowledge this is also the first characterized enzyme specific
17 of the biosynthesis of a cell wall polysaccharide from brown algae.

18

19 **Fine-tuning of alginate structure**

20 In the context of industrial applications, based on various alginates with different physico-
21 chemical properties, the use of specific ManC5-Es that could modulate rheological properties
22 in a defined manner would be of precious biotechnological advantage. Moreover, since
23 significant variations impact the hydrocolloid properties of alginates as a function of seasonal
24 factors, tissue or species, having at hand specific ManC5-Es that could adjust the rheological
25 properties after or during the industrial processing and extraction is also of large interest. In

1 the past, several ManC5-Es of bacterial origin have been biochemically characterized
2 (Ertesvåg 2015, Wolfram et al. 2014, Campa et al. 2004, Hartmann et al. 2002) and even
3 engineered to produce tailored alginates (Tøndervik et al. 2013, Bjerkan et al. 2004). To be
4 useful in existing processes of commercial alginate production, the alginate ManC5-Es need
5 to meet a number of requirements. For example, it is important that the enzymes are selected
6 to work on polymeric substrate, not only on shorter oligosaccharides. This is the case for
7 MEP13-C5, similarly to those of bacterial origin (Campa et al. 2004, Hartmann et al. 2002).

8 In our study, low but significant epimerization activity of the algal MEP13-C5 could
9 be measured by ¹H-NMR and by the indirect measure of the activity of a GG-specific alginate
10 lyase. However at this stage, it remains difficult to compare these preliminary analyses of
11 activity to the mode of action of the bacterial enzymes. All alginate-producing bacteria
12 expressed a periplasmic epimerase, AlgG, and the putative mannuronan C5-epimerases from
13 *Ectocarpus* show highest sequence identity to these bacterial epimerases, with around 32%
14 identity of MEP13-C5, for example, with AlgG from *P. aeruginosa* (Figure 6). Nevertheless,
15 while all characterized bacterial enzymes of the AlgG-type have been reported to be rather
16 calcium-independent (Ertesvåg 2015, Wolfram et al. 2014), the activity of MEP13-C5 could
17 only be measured in presence of Ca²⁺. This situation is reminiscent of the AlgE-type enzymes
18 produced by bacteria of the *Azotobacter* genera, which are calcium-dependent mannuronan
19 C5-epimerases (Wolfram et al. 2014, Hartmann et al. 2002). However the sequences of the
20 algal mannuronan C5-epimerases are much more distantly related to these enzymes, with
21 identities often less than 17%. Despite the lack of sequence similarity and different
22 characteristics, the enzymes of bacterial origins that have been biochemically characterized all
23 display a processive mode of action and common active site residues (Wolfram et al. 2014,
24 Campa et al. 2004, Hartmann et al. 2002), but differences appear with respect to the
25 preference of formation of MG or GG-blocks. Our algal enzyme seems to display a somewhat

1 different specificity than the bacterial enzymes of the AlgG-type, in that it appears to
2 recognize the presence of GG-blocks to preferentially increase the GG block sizes. This
3 observation might be deduced from the NMR study, where the enzyme increased GG-block
4 structures in a substrate already rich in GG-block motifs, but this preference is subtle and not
5 confirmed by the alginate lyase-based assays. A more exhaustive library of standards should
6 therefore be used in future biochemical studies to determine the alginate patterns, which are
7 more susceptible to be epimerized. If verified, this subtle difference in mode of action is not
8 surprising in the view of the differences that can be identified at the primary sequence level,
9 as illustrated in Figure 6, although the characteristic active site signature 'DPHD' and the
10 regular features specific of the β -helix fold align well. In the structural sequence alignment
11 one can notice that several residues directly adjacent to the active site, and identified as being
12 involved in substrate binding and processing in AlgE4 and AlgG (Rozeboom et al. 2008,
13 Wolfram et al. 2014), have a different nature in MEP13-C5 (blue triangles in Figure 6).
14 Moreover, single residue insertions or deletions (green triangles in Figure 6) or large inserted
15 or deleted regions within the catalytic domain (blue dashed lines in Figure 6) may influence
16 the positioning of important residues and influence substrate specificity, which may differ
17 among the different algal ManC-5Es.

18 Studies of the bacterial multigenic C5-epimerase family from *Azetobacter vinelandii*
19 (Hartman et al. 2002, Buchinger et al. 2014) have highlighted the subtle differences in
20 substrate specificities of different AlgE-type enzymes. In the biological context of brown
21 algal cell wall, the presence of a large multigenic ManC5-E family thus points towards the
22 fact that variability in substrate specificity is to be expected. Interestingly, besides the rather
23 well conserved catalytic domain of about 400 amino acids, the modular organisation is highly
24 variable among ManC5-Es from *Ectocarpus*. The functional role of additional R-modules in
25 bacterial C5-epimerases has only recently been analysed (Buchinger et al. 2014) and showed

1 that they do influence alginate-pattern recognition and mode of action. Our study provides a
2 first experimental indication that given their different modular organisation and fine structure
3 at the primary sequence level, the mannuronan C5-Epimerases in brown algae are likely to
4 recognise and/or generate distinct motifs, which are important for the physiological functions
5 and the regulation of alginate synthesis and remodelling in the cell wall. Future structural and
6 biochemical analyses will be necessary to elucidate in detail the potential of fine-tuning
7 alginate structures by these algal ManC5-Es.

8

9 **The roles of ManC5-Es in cell-wall remodelling in brown algae**

10 By acting on a major component of the brown algal cell walls, the ManC5-Es are likely to be
11 of importance in many physiological responses, and various studies are supporting the idea.
12 For instance, in *Laminaria digitata*, some ManC5-E genes are differentially expressed during
13 stress responses such as elicitation and protoplast generation (Tonon et al. 2008), and upon
14 seasonal variations (Nyvall et al. 2003). Probably one of the most important (but the less
15 explored) field of action of ManC5-Es, is during developmental processes. Variations of the
16 alginate structure upon tissues are known in Laminariales species, and our analysis of
17 microarray expression data highlighted the possible role for ManC5-E during the development
18 of gametophyte versus sporophytes. Interestingly, almost all of the ManC5-E genes are
19 constitutively expressed, strongly suggesting that ManC5-Es are major actors during
20 *Ectocarpus* development.

21 In land plants the homogalacturonan motifs of pectins are secreted into the wall as
22 highly methylesterified forms, subsequently modified by the multigene PME family. The
23 PMEs can either act randomly, promoting the action of cell wall hydrolases and contributing
24 to cell wall loosening, or linearly, giving rise to blocks of demethylesterified motifs, which
25 contribute to cell-wall stiffening through calcium chelation (Micheli 2001). In brown algae,

1 the functional analogy of ManC5-Es to PME_s is tempting but challenges our view of their
2 modes of action. In contrast to PME_s in plants, our present knowledge on their epimerase
3 activity indicates a ‘dead-end’ reaction, leading to cell wall strengthening only. The
4 *Ectocarpus* genome does not encode any known alginate lyases, but the presence of enzymes
5 possibly acting on unsaturated oligosaccharides (i.e. GH88; Michel et al. 2010) suggests that
6 new families of polysaccharide lyases are present, or alternatively that some ManC5-Es have
7 the dual activity, although this is not the case of MEP13-C5 (i.e. no lyase activity; data not
8 shown). Also, the analysis of the ManC5-E gene structure in *Ectocarpus* indicates additional
9 modules such as the WSC domains and the AGP-core motifs, which might putatively
10 influence the ManC5-E activity (Hervé et al. 2016). The knowledge of the mechanisms
11 involved in cell wall remodelling in brown algae is still in its infancy, partly because
12 expressing algal enzymes into heterologous systems remains challenging. Nevertheless, our
13 success on this first heterologous and refolded algal C5-epimerase, as well as the access to
14 recent genome sequences (Cock et al. 2010, Ye et al. 2015) should help future analysis on
15 deciphering cell wall metabolism in brown algae.

16

1 **Material and Methods**

2

3 **Identification and bioinformatical analyses of the ManC5-E sequences**

4 *Ectocarpus* contains 31 genes of ManC5-Es, available at the genome website of *Ectocarpus*
5 *siliculosus* at <https://bioinformatics.psb.ugent.be/gdb/ectocarpus> and listed in Supplementary
6 Table S1. For phylogenetic analyses, 10 genes were discarded due to doubtful predicted gene
7 structure. The remaining deduced amino acid sequences encoding the ManC5-E catalytic
8 domains were aligned using the MAFFT program at <http://bioinformatics.uams.edu/mafft/>
9 (Supplementary Fig. S8). Unrooted maximum likelihood phylogenetic trees were conducted
10 using MEGA6 (Tamura et al. 2007). The best-fit model among 48 different amino acid
11 substitution models was the LG model. The tree with the highest log likelihood (-8620.9504)
12 is shown in Figure 1A. Initial trees for the heuristic search were obtained automatically by
13 applying Neighbor-Joining and BioNJ algorithms to a matrix of pairwise distances estimated
14 using a JTT model, and then selecting the topology with superior log likelihood value. A
15 discrete Gamma distribution was used to model evolutionary rate differences among sites (5
16 categories (+G, parameter = 1.1283)). All positions containing gaps and missing data were
17 eliminated. Bacterial sequences were used as an outgroup (Michel et al. 2010). There were a
18 total of 24 sequences and 253 positions in the final data set. The robustness of the branches
19 was evaluated using 100 bootstrap replicates. The modularity of each deduced amino acid
20 sequence was further examined using Blast queries against the UniProt database and domain
21 searches against the InterPro server. Additional refinements were performed manually by
22 examination of the individual HCA plots. Signal peptides and transmembrane domains were
23 predicted using the SignalP and TMHMM servers, respectively. Predictions of chimeric AGP-
24 motifs were performed as described (Hervé et al. 2016). The cluster of ManC5-Es containing

1 modular proteins is described in more details in Figure 1B. For each ManC5-E included in the
2 phylogenetic tree, the gene structure of the full-length sequence is indicated (Figure 1C).

3

4 **Microarray analysis of the ManC5-E mRNA abundances**

5 We explored the microarray dataset that was obtained in Coelho et al. (2011) to carry out the
6 analysis of changes in mRNA abundances of ManC5-E genes in the gametophyte and
7 sporophyte generations (ArrayExpress accession no. E-MTAB-485).

8

9 **Medium throughput screening for cloning and protein expression of the ManC5-Es**

10 The ManC5-E genes were tentatively cloned and heterologously expressed using a medium
11 throughput strategy in a microtiter-plate format, as previously described (Groisillier et al.
12 2010). In short, the open reading frames, trimmed to the catalytic domains only, were
13 amplified by PCR and using primers incorporating specific restriction sites compatible with
14 our ligation strategies (Supplementary Table S2). From 31 starting genes, 12 were discarded
15 due to either doubtful predicted gene structure or incompatibility with our cloning strategy.
16 The amplification products were cloned into the pFO4 vector (a vector modified from pET15b
17 from Novagen, to be compatible with the BamHI/EcoRI ligation strategy) and the pGEX (GE
18 Healthcare Life Science) vectors, encoding an N-terminal His₆-tag and a GST-tag,
19 respectively. Recombinant plasmids were used to transform *E. coli* strains DH5 α (Promega).
20 The validated plasmids were used to transform appropriate *E. coli* strains BL21(DE3) and
21 BL21 (Novagen), respectively. Screening of protein expression was performed at a small-
22 scale range using a 24-well plate format and the auto-inducible ZYP5052 media as described
23 (Groisillier et al. 2010). The plates were centrifuged at 1,200 g for 20 min at 4°C and the
24 pelleted bacteria were resuspended in a lysis buffer containing 50 mM Tris pH 8, 300 mM
25 NaCl, 1 mg/ml lysozyme, 0.1 mg/ml DNase and an anti-proteases cocktail. The cell lysates

1 were further centrifuged at 12,000 g for 20 min. The supernatants contained the soluble
2 expressed fractions. The remaining cell pellets, which contained the insoluble expressed
3 fractions, were extracted with 6 M urea. All fractions were analysed by SDS-PAGE. All
4 further steps were performed on the single insolubly but highly expressed target module of
5 MEP13.

6

7 **Codon optimisation and scale-up expression and purification of MEP13-C5**

8 Codon-optimisation of the encoded catalytic domain of MEP13 (named MEP13-C5) was
9 performed by GeneArt within the framework PolyModE (www.polymode.eu). The sequence
10 was extended with terminal restriction sites for BamHI and EcoRI to be compatible with our
11 cloning strategy (Supplementary Table S2). The codon-optimised sequence was delivered as
12 being cloned into the shuttle vector pMA. To differentiate between the cDNA and the codon-
13 optimized sequence, the latter one is designated *MEP13-C5_{syn}*, where *syn* indicates the
14 synthetic and codon-optimised sequence. *MEP13-C5_{syn}* was excised from the pMA vector
15 using the BamHI and EcoRI restriction enzymes, purified, and further cloned into the pFO4
16 vector. In order to increase our chances of getting recombinant proteins, the codon-optimised
17 sequence was also cloned into the pQE-80L vector (Qiagen). In addition to encode a His₆-tag,
18 this vector contains a PT5 promoter and two *lac*-operators to allow tight regulation of basal
19 level expression, which makes this vector suitable to express toxic genes. The codon-
20 optimised sequence was amplified by PCR from the pMA vector using specific primers
21 (Supplementary Table S2), purified and digested by BamHI and SacI before ligation into the
22 pQE-80L vector. The recombinant plasmids were used to transform *E. coli* strains DH5 α . The
23 plasmid DNA of the candidate clones was purified using Wizard® Plus SV Minipreps DNA
24 Purification Systems (Promega) and the correctness of cloning was confirmed by DNA
25 sequencing.

1 In order to increase our chances of getting recombinant proteins, we decided to use
2 diverse expression strains and included two additional *E. coli* strains developed for the
3 expression of toxic or rare codon containing genes. We therefore transformed *E. coli*
4 BL21(DE3), *E. coli* Rosetta-gami2(DE3)pLysS and *E. coli* BL21CodonPlus(DE3)RIPL cells
5 (all from Novagen) as expression strains. The transformed strains were cultivated in different
6 complex media, including LB and TB (Sambrook and Russel, 2001; Korf et al., 2005). The
7 culture volume ranged from 200 mL to 1 L. To trigger formation of insoluble proteins,
8 induction (1 mM IPTG final) has been carried out permanently at 37 °C overnight at 180 rpm.
9 After 24 hrs, the cells were analysed by microscopy for the presence of inclusion bodies,
10 which contain the insoluble proteins. Cells were harvested by centrifugation at 6,000 g for 30
11 min and mechanically lysed using a French press.

12 The cell lysate was clarified by ultracentrifugation at 100,000 g during 1 hour. The
13 insoluble protein present in the cell pellet was purified and refolded on-column in a single
14 step. For this purpose the pellet was solubilised using a tissue homogeniser and a refolding
15 buffer containing 25 mM Tris-HCl pH 8.0, 200 mM NaCl, 10 mM 2-mercaptoethanol, 10%
16 glycerol, 8 M urea, 40 mM imidazole and 4 mM CaCl₂. The solubilised inclusion body-
17 solution was centrifuged at 100,000 g for 30 min and the supernatant loaded onto a 5 mL
18 HisTrap HP column charged with 100 mM NiSO₄. The refolding on-column was carried out
19 by a slow gradient over two hours against the refolding buffer and using a buffer containing
20 25 mM Tris-HCl pH 7.8, 200 mM NaCl, 3 mM reduced glutathione, 0.3 mM oxidised
21 glutathione, 10% glycerol and 4 mM CaCl₂. The bound proteins were finally eluted in a
22 gradient with the elution buffer containing 25 mM Tris-HCl pH 7.8, 200 mM NaCl, 3 mM
23 reduced glutathione, 0.3 mM oxidised glutathione, 10% glycerol, 500 mM imidazole and 4
24 mM CaCl₂. Protein purification was carried out on an Äkta Avant Chromatographic System
25 from GE Healthcare. Columns and resins used were from GE Healthcare. All fractions

1 collected were analysed by SDS-PAGE. The targeted eluates containing the refolded MEP13-
2 C5 were pooled and the volume was reduced by ultrafiltration to approximately 3.5 mL of a
3 final protein concentration of 1.94 g/L. In the aim to separate the putative different folding
4 intermediates, a second purification step was performed by size exclusion chromatography
5 using a Superdex 200 column from GE Healthcare. Equilibration and running buffers were
6 those that were used in the final step of the refolding procedure.

7

8 **Western-blotting**

9 The sequences of the catalytic domains of all the brown algal ManC5-Es were aligned with
10 the sequence of the catalytic domain of AlGE4 from *Azetobacter vinelandii* in order to identify
11 specific sequence stretches that, when reported onto the structural coordinates of AlGE4,
12 appeared to be located at the surface of the protein. Two stretches
13 (CDGRAKNEMGECRMDIINS and NHGIIASKRCNNVKIF) were identified and polyclonal
14 antibodies raised in mice against these peptides were purchased from Eurogentech. The
15 antibodies were tested by ELISA and further used to verify heterologous expression of algal
16 ManC5-Es (Supplementary Figure S3).

17

18 **Analysis of protein identity by peptide mass fingerprinting**

19 The refolded and purified MEP13-C5 was analysis on a SDS-PAGE gel. Bands at 50 kDa,
20 which correspond to the expected size of the protein, were excised and analysed by peptide
21 mass fingerprinting (PMF). Coomassie blue-stained protein slices were cut into small pieces,
22 washed with distilled water and de-stained using acetonitrile. The cysteine residues were
23 reduced by 100 µl of 10 mM DTT at 56°C and alkylated by 150 µl of 55 mM iodoacetamide
24 at room temperature. Iodoacetamide solution was replaced by 100 µl of 100 mM NH₄HCO₃
25 and gel dehydration was achieved with acetonitrile. After evaporation in Speed Vac

1 (Thermo), proteins were digested overnight at 37°C by a solution containing 0.9 µg of a
2 modified bovine trypsin (Promega, Madison, WI, USA) prepared in 25 mM NH₄HCO₃.
3 Finally, a double extraction was performed, first with 1% (v/v) trifluoro acetic acid solution,
4 and subsequently with 100% (v/v) ACN. The resulting peptide mixture was extracted and
5 dried under vacuum and re-suspended in 50% ACN, 0.1% TFA solution. PMF by MALDI-
6 TOF MS was performed using a Voyager DE-STR MALDI-TOF mass spectrometer (Applied
7 Biosystems). Each pooled tryptic digest (1 µL) was mixed with an equal volume of CHCA
8 matrix (Sigma; 10 mg/mL in 50% ACN, 0.1% TFA) and spotted onto the MALDI target.
9 Spectra were acquired in positive ion reflector mode under 20 kV accelerating voltage and a
10 mass range of 450– 5000 Da. Internal calibration was performed using trypsin autolysis
11 fragments at m/z 1433.70, 2163.05, and 2289.1. The samples resulting from attempts to
12 produce soluble proteins mostly contained proteins from the *E. coli* host strains, while the
13 samples containing insoluble proteins released eighteen peptides specific of MEP13-C5_{syn},
14 covering 52% of the protein sequence (Supplementary Figure S5), and there was no
15 contamination with *E. coli* proteins. This result demonstrated that MEP13-C5 was indeed
16 highly expressed in an insoluble form, whereas each attempt to produce the enzyme in its
17 soluble form failed.

18

19 **Dynamic light scattering (DLS)**

20 DLS was performed to control the mono-dispersity of the refolded protein. 100 µL of sample
21 proteins (unfolded; refolded fractions) were centrifuged at 20,817 rcf on an Eppendorf
22 Centrifuge 5417R for 10 minutes. 60 µL of supernatant was transferred into a quartz cuvette
23 and placed into a Zetasizer Nano S from Malvern. Measurement followed at 20°C during 20
24 minutes.

25

1 **Enzyme activity assays**

2 All alginate samples were provided by DuPont. 100 μg of sample proteins were incubated in
3 presence of 0.5% sodium alginate (M/G ratio of 1.5 and 1.7) in a buffer containing 20 mM
4 MOPS pH7.0, 4 mM CaCl_2 , 100 mM NaCl, using a final volume of 1 mL. The reaction was
5 incubated overnight at 30°C. To avoid signal-interference in subsequent NMR measurements,
6 it was necessary to remove the CaCl_2 after the enzymatic reaction. Removal of CaCl_2 started
7 with acidification of the samples by mixing them with equal volume (1 mL) of acidic water
8 (300 μL of concentrated H_2SO_4 in 100 mL of water, pH 1.4) and incubation for one hour at
9 room temperature on a shaker. The samples were centrifuged at 141 g for 5 minutes and the
10 top phase was removed. The samples were washed with an equal volume of water (1 mL) and
11 incubated in the same way as before. Each washing step with acidic water and normal water
12 was done three times. In the final step, the top phase (water) was removed and the samples
13 were neutralised with Na_2CO_3 (0.2 g in 10 g water) to a pH of 7.0. MEP13-C5 untreated
14 alginates were submitted to the same procedure as the MEP13-C5 treated samples. The
15 samples were lyophilized prior to measurements. Immediately before performing NMR
16 measurements the samples were solubilized with D_2O . NMR-spectra were measured without
17 depolymerisation of the samples.

18 Non-lyophilized samples, without calcium removal, after treatment with refolded
19 MEP13-C5 were also analysed in a second, indirect, activity assay using the action of a G-
20 specific alginate lyase known as AlyA1 from *Zobellia galactanivorans* (Thomas et al. 2013).
21 The MEP13-C5 treated alginate samples were incubated at 30°C with 100 μg of AlyA1 in a
22 solution containing 100 mM Tris-HCl buffer at pH 7.0. Alginate lyase activity was assayed by
23 measuring the increase in absorbance at 235 nm (A_{235}) of the reaction products (unsaturated
24 uronates) for 5 min in a 1 cm quartz cuvette containing 0.5 ml of reaction mixture in a

1 thermostated spectrophotometer. The difference in the AlyA1 activity between the MEP13-
2 C5-treated and -untreated alginates was assigned to the MEP13-C5 activity.

3

4 **NMR analysis**

5 The untreated and enzyme-treated alginate samples were prepared as described above. All
6 NMR experiments were performed on a Bruker Avance III spectrometer (Karlsruhe,
7 Germany) equipped with a double-tuned direct-detection broadband 5 mm probe (and
8 deuterium lock) and a 14.1 T magnet corresponding to a ^1H resonance frequencies of 600
9 MHz. Each spectrum was run with a pulse angle of 30° , 2.7 second acquisition and one
10 second delay between each scan with a total of 64 scans. The data was zero filled to 65536
11 and a Gaussian apodiazion function was added prior to the Fourier transform yielding a
12 spectrum with a 20 ppm spectral width. Further processing such as deconvolution, was done
13 using python scripting in combination with the nmrglue python library (Helmus et al. 2013).
14 The chemical shifts are expressed in parts per million (ppm) downfield from the signal for 3-
15 (trimethylsilyl)propanesulfonate.

16 For the alginate substrate with a M/G ratio of 1.7, four control samples were
17 measured, giving an average M/G ratio of 1.68 and a standard deviation (sd) of 0.02. When
18 the enzyme was added (4 replicates) the average M/G ratio decreased to 1.51 with an sd of
19 0.01. For the alginate substrate with a M/G ratio of 1.5, four control samples were measured,
20 giving an average M/G ratio of 1.46 and a standard deviation (sd) of 0.01. When the enzyme
21 was added (4 replicates) the average M/G ratio decreased to 1.33 with a sd of 0.02. The
22 measure of block distribution in terms of “GG”, “MM”, ”GM = MG ”, ”GGG”, “MGM”,
23 “GGM = MGG”, was undertaken by deconvolution or integration of the peaks in the region
24 between 4.40 and 5.15 ppm following standard protocols (Grasdalen et al. 1981, Grasdalen
25 1983, Hartmann et al. 2002).

1

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6 National de la Recherche Scientifique (CNRS).

7

1 **Abbreviations**

- 2 ManC5-E, mannuronan C5-epimerase
- 3 MEP, mannuronan epimerase
- 4 M, β -D-mannuronic acid
- 5 G, α -L-guluronic acid
- 6 PME, pectin methylesterase
- 7 AGP, arabinogalactan protein
- 8 WSC, Wall Sensing Component
- 9 Tris, Tris(hydroxymethyl)aminomethane or 2-Amino-2-hydroxymethyl-propane-1,3-diol
- 10 MOPS, 3-(N-morpholino)propane-1-sulfonic acid
- 11 ACN, acetonitrile
- 12 TFA, trifluoroacetic acid
- 13 CHCA, α -cyano-4-hydroxycinnamic acid
- 14 DTT, dithiothreitol
- 15 IPTG, isopropyl β -D-1-thiogalactopyranoside
- 16 Sd, standard deviation
- 17 HCA, hydrophobic cluster analysis
- 18 PMF, peptide mass fingerprinting
- 19 MS, mass spectrometry
- 20 MALDI-TOF, matrix-assisted laser desorption/ionisation – time of flight spectrometry
- 21 ELISA, enzyme-linked immunosorbent assay
- 22 SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis
- 23 DLS, dynamic light scattering
- 24 NMR, nuclear magnetic resonance
- 25 Ppm, parts per million

1 **Legends to figures**

2

3 **Fig. 1.** Schematic representation of the effect of ManC5-Es on alginate structures. Alginate in
4 brown algae is first formed as a polysaccharide chain containing mannuronic acid residues
5 only; these are subsequently transformed by the ManC5-E into guluronic acid residues,
6 generating distinct patterns arranged in regions of MM-, GG- and MG-blocks (top). Patterns
7 containing large stretches of adjacent guluronic acid residues (GG-blocks) form structured
8 inter-chain associations in the presence of Ca^{2+} ions (central). These inter-chain junctions
9 have the so-called ‘egg-box’ conformation (bottom left) and are responsible for the gelling
10 properties of alginate and cell-wall strengthening.

11

12 **Fig. 2.** Bioinformatical analyses of the ManC5-E family from *Ectocarpus*. **(A)** Phylogenetic
13 tree. Numbers indicate the bootstrap values in the maximum likelihood analysis. The
14 sequences belonging to the four main clusters are marked by distinctive symbols (black
15 diamond, open circle, black square and black triangle). **(B)** A focus is made on the ManC5-E
16 sequences marked by an open circle. Putative structure of the encoded proteins is shown on
17 the right side with the signal peptide (black box), the catalytic domain ManC5-E, the WSC
18 domains, the AGP protein-core featuring the glycan decoration (green “wattle blossom”
19 shape), the module of unknown function (yellow oval) and a transmembrane domain (red
20 box). Lengths are relatively scaled. **(C)** Corresponding gene intron-exon structure of the
21 ManC5-Es shown in the tree.

22

23 **Fig. 3.** Microarray analysis of the abundance of ManC5-E transcripts in *Ectocarpus*
24 sporophytes versus gametophytes, obtained from Coelho et al. (2011). The belonging to the
25 clusters described in the phylogenetic tree shown in Figure 2A is indicated (bottom). Data are

1 means of three independent biological replicates \pm SE. Asterisks indicate significant
2 differences (t -test, $P < 0.05$).

3

4 **Fig. 4.** Assessment of mannuronan C5-epimerase activity of MEP13-C5 by ^1H -NMR. (A) The
5 average M/G ratio of two different industrial alginates (M/G ratios of 1.5 and 1.7) before and
6 after treatment with the recombinant and refolded MEP13-C5 are displayed as histograms. (B)
7 Average G-block length of the corresponding samples. (C) The relative occurrences of the
8 distinct block structures of the alginate samples are shown. The scale of the x-axis
9 corresponds to block structures derived from NMR data by integration and deconvolution of
10 individual NMR peaks in the region of 4.40 to 5.15 ppm. In each case, data are means of four
11 independent replicates \pm SD. Asterisks indicate significant differences (t -test, $P < 0.01$).

12

13 **Fig. 5.** Assessment of mannuronan C5-epimerase activity of MEP13-C5 by analysis of the
14 degradation pattern using a GG-specific bacterial alginate lyase. The production of
15 unsaturated sugar units produced by the action of AlyA1 (a strictly GG-specific alginate lyase
16 from *Z. galactanivorans*) was followed during 5 minutes by photospectrometry at a
17 wavelength of 235 nm on the two alginate samples (M/G ratios of 1.5 and 1.7). In each case,
18 data are means of three independent replicates \pm SD.

19

20 **Fig. 6.** Extract of a structural sequence alignment of selected mannuronan C5-epimerases
21 from *Ectocarpus*, including MEP13, against the sequence and structural features of the
22 bacterial AlgG (pdbcode 4NK6). One sequence from each cluster of the phylogenetic tree
23 shown in Figure 2A (namely MEP5, MEP13, MEP25 and MEP29) were used and the ManC5-
24 E catalytic sites were aligned against the sequence of AlgG (pdbcode 4NK6) from
25 *Pseudomonas aeruginosa*. The alignment was edited using the program ESPRIPT (Robert and

1 Gouet, 2014). The colour code of the amino acid residues is according to their biochemical
2 character. The secondary structure assignments and residue numbering above the sequences
3 correspond to those of 4NK6. Regions displaying high sequence similarity are boxed. The red
4 triangles below the sequences indicate the catalytic residues His319 and Asp320, the blue
5 triangles below the sequences indicate two positions in this highly conserved pattern that are
6 modified in the algal ManC5-Es, the green triangles highlight a single residue
7 insertions/deletions close to the active site and the blue dashed lines highlight larger
8 insertion/deletion between the algal clades and the bacterial sequence.
9

1

2 **Tables**3 **Table 1.** Statistics for several features of the ManC5-E genes, including MEP13-C5. **(A)**4 Structure of genes compared to the *Ectocarpus* genome, **(B)** relative adaptiveness values (%)5 for 5 rare codons for *E. coli*, compared to the nuclear codon usage of *E. coli*

A				B					
	genome (average)	ManC5-Es (average)	MEP13		AGA	AGG	ATA	CTA	CCC
Gene length (bp)	6,974	9,712	5,494	<i>E. coli</i> (genome)	43	23	45	16	43
CDS length (bp)	1,607	2,083	1,899	ManC5-Es (average)	40	60	9	11	79
Number of introns per gene	7.1	13.7	10.0	<i>MEP13-C5</i>	33	100	4	0	91
				<i>MEP13-C5_{syn}</i>	0	0	0	0	0

6

7

8

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